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DNA Analysis of Surfactant Associated Bacteria in the Sea Surface Microlayer in Application to Satellite Remote Sensing Techniques: Case Studies in the Straits of Florida and the Gulf of Mexico

By

Bryan Hamilton

Submitted to the Faculty of

Nova Southeastern University Oceanographic Center

in partial fulfillment of the requirements for

the degree of Master of Science with a specialty in:

Marine Biology

Nova Southeastern University

21 May 2015

ACKNOWLEDGEMENTS

I thank my major advisor Dr. Alexander Soloviev for giving me the opportunity to work on this research while in his lab and for all of his guidance throughout my time at Nova Southeastern University. I am also grateful to my committee members, Drs. Aurelien Tartar, Mahmood Shivji, and Brian Haus for sharing their expertise and advice throughout my project. I thank Dr. Tartar for helping throughout the DNA analysis stage of the project and for letting me use his laboratory and equipment. I thank Dr. Shivji for his contributions to the final paper and for sharing his expert advice in genetics. I also thank Dr. Haus for his contributions to the paper and for his much needed assistance during sampling trips. He helped organize sample collection during the SCOPE experiment in the Gulf of Mexico. I am grateful to David Ortiz-Suslow (UM), Nathan Laxague (UM), Brian Ettinger (NSU) and Michael Choron (All Jacked Up Gulf Coast Fishing Charters), whom assisted with sample collection in the field. I thank Cayla Dean for all of her help with sample collection as well as DNA analysis and Katrina Finns for her assistance in the lab throughout the project.

I acknowledge support from the GoMRI project "Consortium for advanced research on transport of hydrocarbon in the environment" (PI: Tamay Özgökmen, UM RSMAS). Funding for this project was also provided through the Nova Southeastern University (NSU) President's Faculty Research and Development Grant.

Finally, I thank my family and friends for their continued encouragement and support throughout both my undergraduate and graduate studies.

ABSTRACT

Several genera of bacteria residing in the sea surface microlayer and in the near-surface layer of the ocean have been found to be involved in the production and decay of surfactants. Under low wind speed conditions, surfactants can suppress short gravity capillary waves at the sea surface and form natural sea slicks. These features can be observed with both airborne and satellite-based synthetic aperture radar (SAR). Using a new microlayer sampling method, a series of experiments have been conducted in the Straits of Florida and the Gulf of Mexico in 2013 to establish a connection between the presence of surfactant-associated bacteria in the upper layer of the ocean and sea slicks. In a number of cases, sampling coincided with TerraSAR-X and RADARSAT-2 satellite overpasses to obtain SAR images of each study site. Samples collected from slick and non slick conditions have been analyzed using real time PCR techniques to determine *Bacillus* relative abundance in each area sampled. Previous work has shown that the sea surface microlayer plays a role in air-sea gas exchange, sea surface temperature, climate-active aerosol production, biochemical cycling, as well as the dampening of ocean capillary waves. Determining the effect of surfactant-associated bacteria on the state of the sea surface may help provide a more complete global picture of biophysical processes at the air-sea interface and uptake of greenhouse gases by the ocean.

Keywords: bacteria, synthetic aperture radar, real time PCR, sea surface.

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1. Introduction

1.1 Sea surface microlayer

The sea surface microlayer is defined as the thin biogenic layer that is present at the sea surface-atmosphere boundary (Liss and Duce 1997). The microlayer is thought to make up the uppermost 1 mm of the ocean, although some research has estimated a depth less than 50 μm (Franklin et al. 2005). Previous work has shown that this microlayer plays a role in air-sea gas exchange, sea surface temperature, climate-active aerosol production, biochemical cycling, as well as the dampening of ocean capillary waves (Upstill-Goddard 2003; Wurl et al. 2011). The microlayer is made up of many materials that have accumulated at the ocean surface, primarily lipids, polysaccharides, proteins, and surfactants (Norkrans 1980). Traditionally, it was thought that the microlayer was somewhat structured, as seen in Figure 1 (Norkrans 1980; Hardy 1982). However, it is now believed to be more of a complex matrix of dissolved organic materials that are produced in the marine environment (Sieburth 1983; Wurl and Holmes 2008).

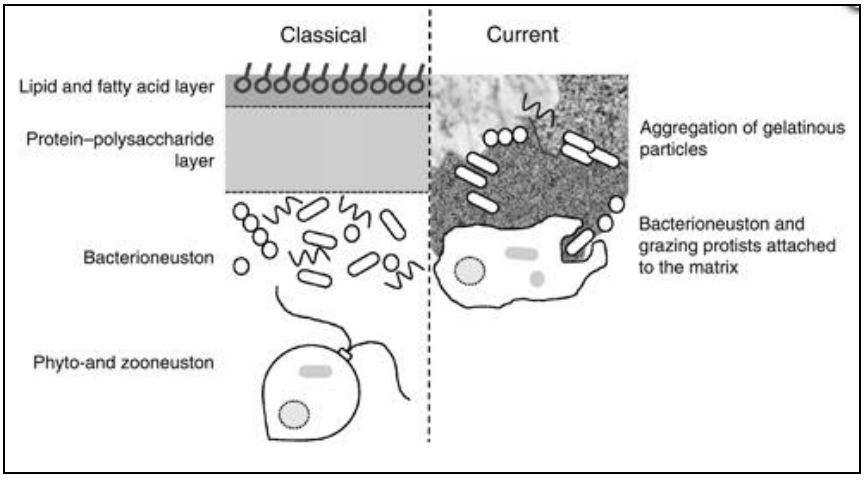

Figure 1. Classical and current view of the sea surface microlayer. From Cunliffe et al. 2011.

Surfactants found in the microlayer are amphiphilic, meaning that they have both a hydrophilic and hydrophobic portion (Desai and Banat 1997). Because of this structure, surfactants are present at the interface between air and water. When surfactants along with other materials accumulate on the sea surface, they can suppress ocean capillary waves and form natural sea slicks in low wind speed conditions. These slicks can be visible to the naked eye, and are also seen from synthetic aperture radar (SAR) satellite imagery. SAR can be also used to observe many other ocean surface processes, such as oil spills, coastal discharges, algal blooms, as well as ship traffic (Liew 2001).

1.2 Microorganisms in the marine environment

Microbes found in marine environments are vital to life on this planet, as it most likely began in the oceans (Karl 2007). This would make marine microorganisms the closest living descendants to some of the first forms of life. Marine microbes are known to carry out many steps of the biogeochemical cycles other organisms aren't able to complete. The ocean is dominated by them in both biomass and metabolism. They are very helpful in marine food webs, as they are able to feed on dissolved organic matter and help transfer energy back into food chains. Microbes are found in all marine ecosystems, from the deep abyss to the sea surface and from the tropical regions to the sea ice found close to the poles (Karl 2007). Many forms can consume greenhouse gases such as carbon dioxide and methane and are vital to the ocean's carbon cycle.

There is an estimated 3.67×10^{30} microorganisms in marine environments, indicating a massive amount of diversity. However, most of the microbial world in the ocean remains unexplored. According to some estimates, less than 0.1% of these

microbes are known today (Whitman et al. 1998). Part of the reason that there is so much unknown in the field of marine microbiology is because many of these organisms are not culturable in the lab (Harayama et al. 2004). In recent years, a number of analysis tools such as next generation sequencing and real time PCR have been created to begin to gain more of an understanding of marine microbes and the processes they are involved in.

1.3 Microbial activity in the microlayer

There have been a limited number of studies regarding microbial life that is found in or near the sea surface microlayer. The term 'neuston" is currently used to describe organisms that live in the microlayer (Cunliffe and Murrell 2009). This environment can benefit bacterioneuston due to the gelatinous dissolved organic materials that accumulate at the surface. Microbes can colonize and obtain nutrients necessary for survival (Maki 2002). However, this is also an extreme environment with fluctuating temperatures and salinity, pollutant accumulation, as well as solar and UV radiation. Certain types of bacteria may be better suited for this environment, such as those that produce surfactants. When bacterioneuston aggregate at the surface, high nutrient hot spots can form, which cause an increase in biological activity compared to the subsurface water (Hardy 1982).

1.4 Surfactant associated bacteria

Surfactants are produced and degraded by a number of organisms in the marine environment. While certain types of phytoplankton are known to be a major contributor to surfactant production, bacteria are also known to produce these materials. These microbes have been isolated from both terrestrial and marine environments (Satpute et al. 2010). Genera such as *Acinetobacter*, *Pseudomonas*, *Bacillus*, *Enterobacter*, and

Corynebacterium have all been reported to produce surfactants (Satpute et al. 2010; Abraham et al. 1998; Maneerat et al. 2006; Perfumo et al. 2006). Bacteria produce these materials for a number of reasons, including aggregation, protection, and to utilize nutrients more efficiently (Kurata 2012; Sayem et al. 2011; Paul et al. 2007).

1.5 Previous work on microbes in the sea surface microlayer

A small number of studies have been done that compare communities of bacteria found in the sea surface microlayer and the subsurface waters directly below, with somewhat conflicting results. The sampling methods vary between each study, highlighting the need for a microlayer sampling standard that can be used across the scientific community. Multiple studies could then be compared more directly without the bias associated with each type of sampling method used today (Cunliffe et al. 2013)

Franklin et al. (2005) sampled bacterial communities from the microlayer as well as underlying subsurface water in the North Sea from 1999 to 2000. Microlayer samples were collected using polycarbonate membrane filters 47 mm in diameter. The filters were placed on the surface for 10 seconds and retrieved using sterile forceps. Subsurface samples were collected by pumping water from 1 m depth into glass tight bottles. DNA from these samples was then extracted, at which point phylogenetic analysis took place. Results showed a difference in community structure between microlayer and subsurface samples. Microlayer samples were less diverse than those from the subsurface, being dominated by two major groups: *Vibrio* ssp (68%) and *Pseudoalteromonas* (21%). Results point to a distinct bacterioneuston population that differs from communities in subsurface waters.

A related study was performed by Agogue et al. (2005a), who compared microlayer and subsurface samples collected in the Mediterranean Sea from 2001 to 2002. Samples were collected at two sites, each with very different water conditions. One area was found in polluted waters heavily impacted by urban sewage outfall, while the other area was located in oligotrophic waters. Multiple methods were used to collect from the microlayer, all having different sampling depths and collection volumes. A metal screen and glass plate were the primary devices; however a nylon screen, a Harvey roller, and polycarbonate membrane filters were also used. No significant differences in bacterial species were found using these methods. For subsurface samples, a bottle was submerged in water to 0.5 m depth. The bottle was then opened underwater to collect each sample.

Culturing as well as genetic fingerprinting methods were used to compare the communities from these two environments. *Proteobacteria* was found to be more abundant in the oligotrophic site, whereas *Actinobacteria* and *Firmicutes* were more abundant in the polluted area. However, there were no significant differences between communities in the microlayer samples compared to subsurface communities. This study suggested that stable bacterioneustonic communities may not be common in coastal environments.

A separate study performed by Agogue et al. (2005b) focused on the resistance levels to solar radiation by bacteria found at the sea surface and underlying waters. Sampling took place again in the Mediterranean Sea and multiple techniques were used to collect the bacteria. These methods included the metal screen, the glass plate, and the nylon screen. Bacteria types were indentified using sequence analysis and growth

recovery was looked at after exposing the bacteria to certain levels of simulated solar radiation. This study found that the resistance levels of microlayer bacteria did not differ significantly from subsurface communities. The authors stated that polysaccharide production by surface bacteria may protect them from the levels of radiation at the surface of the ocean.

Cunliffe et al. (2008) performed a study on microbial communities found in the Blyth River tidal estuary, which feeds into the North Sea. Samples were taken at two sites with different salinities to determine if communities would differ due to that factor. The polycarbonate sampling method previously used by Franklin et al. (2005) was used for this study. Polycarbonate membrane filters were deployed on the sea surface and collected using sterile forceps. Subsurface water was collected by placing a bottle 0.4 m in depth and opening the lid. Diversity and community structure was determined by denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene PCR amplicons. Cunliffe et al. discovered that the communities found in different salinities were very similar and that there were no bacterial groups that dominated a certain area. This study also showed differences in community structure when comparing microlayer and subsurface samples.

A more recent study by Lindroos et al. (2011) focused on comparing microlayer bacteria to subsurface bacteria in the Baltic Sea. Using a mesh screen and membrane filters, they reported a higher abundance of bacteria in the microlayer. The studies that have been done on microbial communities found in the microlayer show the need for a standard sampling method for the scientific community to use. They also show the need

to continue to study this environment, as microlayer community research is still in its infancy.

Kurata et al. (2012) took microlayer samples in the Straits of Florida using polycarbonate membrane filters. A goal of the project was to design a sampling technique that could be easily standardized and would limit contamination as much as possible. The technique used to obtain bacteria was used in the current study and is described in the materials and methods section below. Subsurface samples were collected using an environmental water sampler to compare surfactant associated bacteria between the two depths. Samples were taken in both slick and non slick conditions to see how the bacterial community differed as well. Collection coincided with a SAR satellite overpass to obtain images of the slicks that were present. Kurata used next generation 454 sequencing technology to determine bacterial community structure. Results from that study showed a high abundance of known surfactant associated genera in slick subsurface waters (Fig 2).

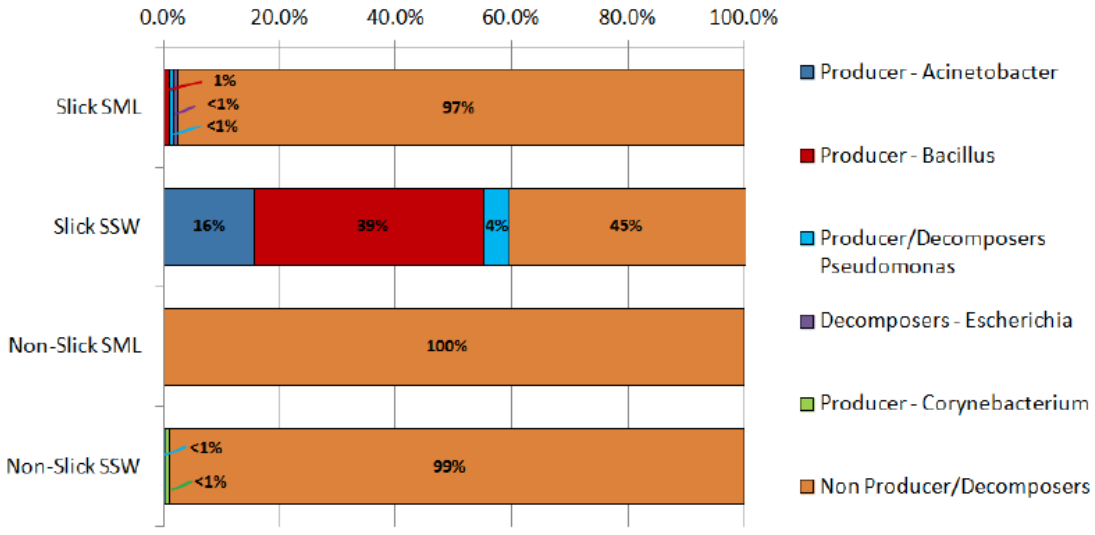

Figure 2. Abundance levels of surfactant associated genera of bacteria from four samples analyzed in Kurata's (2012) study.

In Kurata's (2012) study, *Bacillus* was shown to be more abundant in slick conditions than in non slick waters, pointing to a possible connection between the presence of this group of bacteria and sea slicks. It was also mostly found in the water column below the slick. The findings of this case study suggest that surfactant associated bacteria may be residing in the water column and surfactants that they produce are being transported up to the sea surface by physical processes, contributing to slick formation. This possibility has not been addressed in other microbial studies of the near surface layer of the ocean and will be addressed below. Bacteria belonging to the genus *Bacillus* are a very diverse group of organisms, with many marine isolates having the ability to produce structurally diverse metabolites such as polypeptides, fatty acids, lipopeptides, and lipoamides (Hamdache et al. 2011; Baruzzi et al. 2011). These compounds exhibit a range of biological uses, such as antimicrobial and antialgal capabilities. Members of *Bacillus* can be found in almost every niche of both terrestrial and marine environments. A few species have already become very important for industrial applications due to their ability to produce surfactant materials (Banat et al. 1995a; Banat et al. 1995b; Rodrigues et al. 2006). *Bacillus* was also shown to not be a source of contamination in control filters taken during that study. For these reasons, this genus of bacteria became the focus of the current study.

1.6 Sea surface microlayer and SAR

Synthetic aperture radar (SAR) satellite imagery can be used for a variety of applications to monitor the environment. Specifically, it can be an effective tool for seeing features on the sea surface (Wiley 1985). This technology requires an antenna to be mounted on a platform such as a satellite. The device transmits microwave pulses

towards the target area and the energy that reflects back to the satellite is then measured (Fig. 3) (Liew 2001).

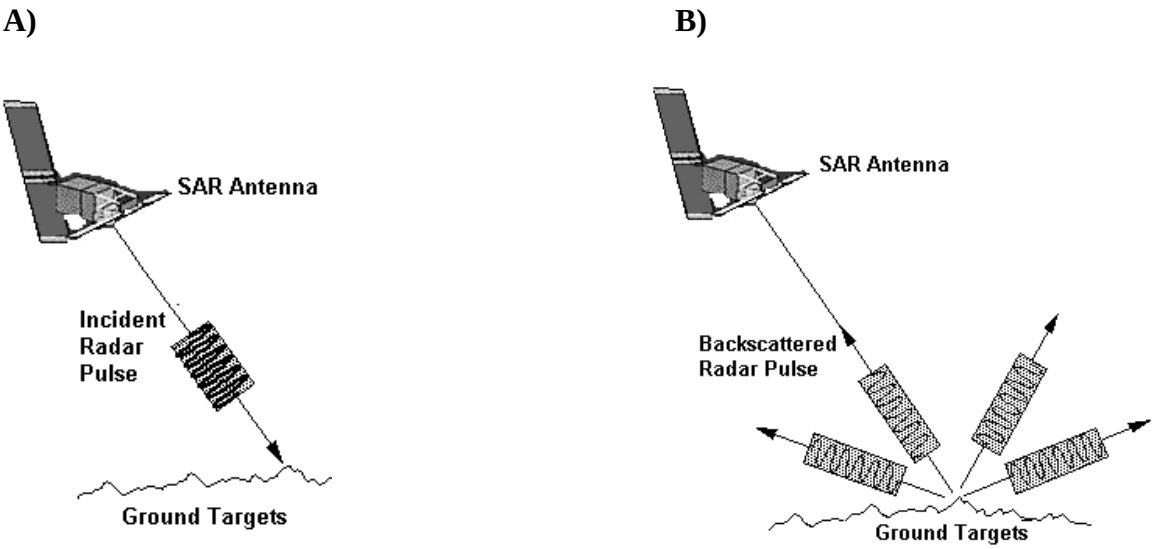

Figure 3. Synthetic Aperture Radar (SAR) antenna. a) The antenna first transmits a radar pulse to the ground. b) The radar pulse is scattered by the ground target and any microwave energy directed back at the antenna is measured. From Liew 2001.

If there is considerable roughness on the sea surface, the energy is reflected away in every direction. The SAR sensor will receive part of this scattered energy, causing a brighter appearance on the sea surface image. If the surface is smooth, the majority of the radar energy is reflected away, leading to a dark appearance in the image (Fig. 4). Using this technology, fine-scale features on the ocean surface can then be captured. Some features that can be seen on the ocean surface using SAR include ship wakes, oil slicks, as well as natural sea slicks. Surfactants produced by a number of organisms including bacteria may thus have a significant influence on fine scale SAR satellite imagery. While satellite remote sensing has been used to observe slicks on the sea surface, little research focusing on a possible connection between marine bacteria, the production of surfactants, and features seen in SAR imagery has been done up to this point (Gade et al. 2006; Espendal and Johannessen 1996).

A) Smooth sea surface **B) Rough sea surface**

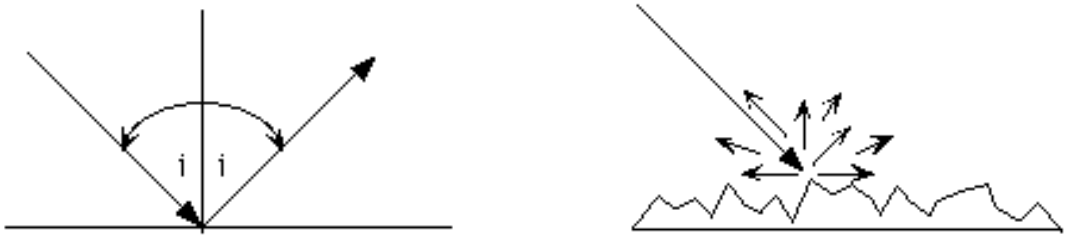

Figure 4. SAR microwave scattering. a) Reflection of the microwaves off of a smooth surface will produce a dark area in SAR imagery. b) Microwaves scattering off of a rough surface will produce a brighter area. From Liew 2001.

1.7 Polymerase Chain Reaction

Real time Polymerase Chain Reaction (PCR) is based on the ground-breaking method of PCR, which was first developed by Kary Mullis in the 1980s (Valasek and Repa 2005; Mullis 1990; Mullis and Faloona 1987). PCR enables researchers to amplify specific pieces of DNA by over a billion fold using DNA polymerases and short, sequence specific oligonucleotides called primers. This process involves the cyclic heating and cooling of a reaction containing DNA. During the heating phase, double stranded DNA is "melted" into single strands. When the reaction is cooled, the oligonucleotide primers that are also in the reaction mix anneal to a specific location on the now single stranded DNA template. The DNA polymerase enzyme then begins the elongation process by adding single nucleotides that are complementary to the single stranded template. This creates a new double stranded DNA copy (Powledge 2004). The process of heating and cooling is then repeated a number of times, each time creating double the amount of DNA (Fig. 5). These new copies of DNA are called amplicons, and they have a number of uses depending on the research performed.

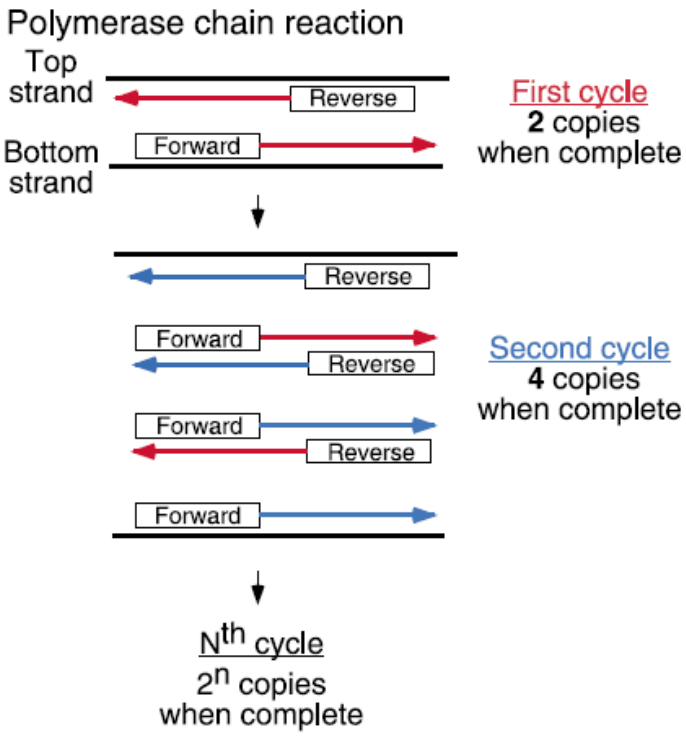

Figure 5. PCR depiction showing the heating and cooling of a DNA sample. When heated, the double strand splits into a top and bottom strand. When cooling takes place, primers attached to each strand and DNA polymerase begins to synthesize a complementary strand, forming a double stranded copy of the original double strand. From Valasek and Repa 2005.

As the PCR reaction runs its course, the efficiency of amplification after each cycle will eventually lessen and reach a plateau level. This plateau effect can be due to a number of reasons, such as the exhaustion of materials in each PCR reaction mix (e.g. primers) or inhibitors within the sample tube. The leveling off of amplification poses a problem if the researcher wants to determine the starting abundance of a target sequence of DNA. Even if there are different amounts of target sequences in a number of reaction tubes to begin a PCR run, there can be similar amounts of amplified DNA after the PCR reaction ceases and any differences in abundance present at the beginning of the run are lost.

Real time PCR solves the issue described above by enabling researchers to monitor the amplification taking place in each sample tube as it is happening using

fluorescence. It has been known since the 1960s that certain fluorescent dyes, such as ethidium bromide, increases fluorescence when they bind to nucleic acids. Combining this chemistry with PCR and real time video recording led to the start of real time PCR in the early 1990s (Higuchi et al. 1992). Present day real time PCR methods usually involve fluorogenic probes that "light up" to show the amount of DNA present after each cycle of PCR is completed. A dye often used today is called SYBR green I, which binds to minor grooves of DNA and emits 1000 fold greater fluorescence than when it is not bound to any product in a solution. Because of this, the more DNA that is in the reaction tube, the greater the amount of DNA binding and fluorescence being produced from SYBR green I. This fluorescence is then measured by the real time PCR machine using lights or lasers, depending on the model.

Software platforms simplify the analysis of real time PCR data by creating a graphic output of the amplification process (Fig. 6). The graph shows the moment when each sample being analyzed reaches the linear phase of amplification and when it comes to the plateau phase described earlier. A threshold level is chosen automatically by the software using a number of algorithms and the cycle numbers when the samples are in their linear phases of amplification. The researcher can also change this threshold level if needed. The cycle count at which a sample crosses the threshold is labeled as its CT value, and this number can then be used for quantitative or relative abundance comparisons between samples.

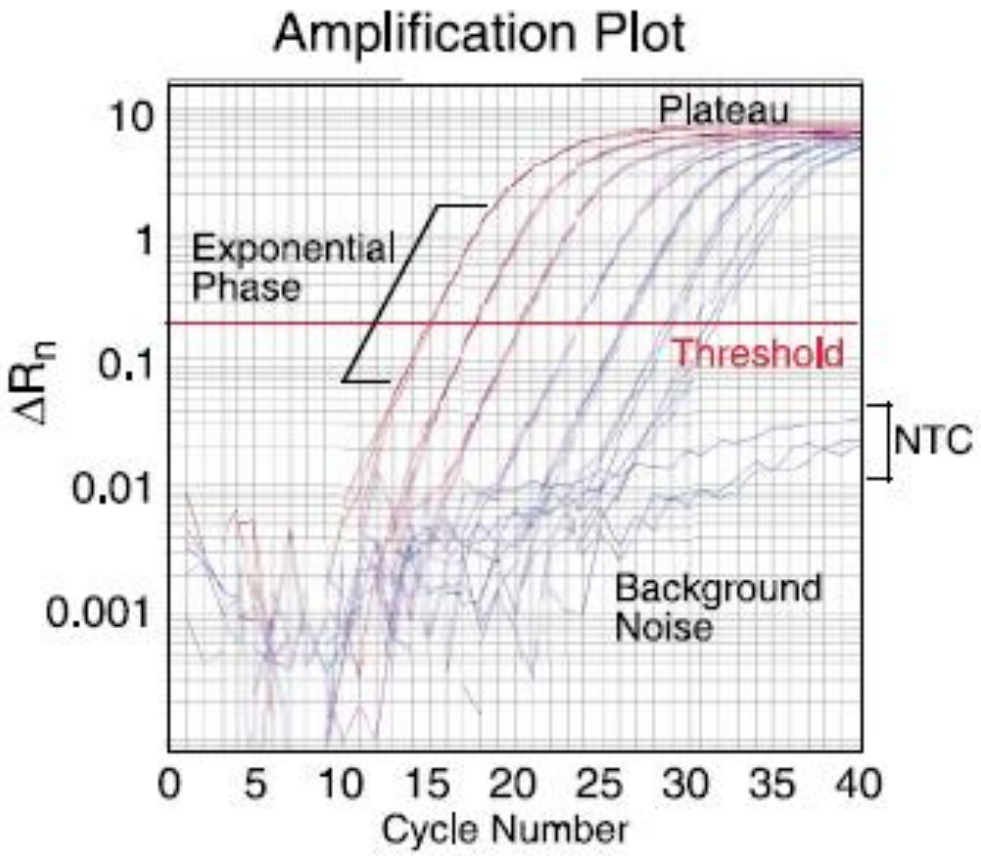

Figure 6. Graph representation of real time PCR run, showing the exponential phase, the plateau phase, as well as typical lines representing background noise. The y axis represents the reporter signal normalized to a passive reference dye. NTC stands for no template control, which is a reaction mix without any DNA template. If the NTC shows the exponential phase similar to the other lines in the graph above, that would be a sign of contamination or incorrect amplification. From Valasek and Repa 2005.

The benefits of real time PCR have been immense in a number of scientific fields. The method has been shown to detect very small quantities of target sequences, making it possible to analyze small samples in clinical studies or from the environment (Valasek and Repa 2005). Real time PCR has the ability to distinguish these specific sequences from complex mixtures of DNA. Many systems also include melt curve analysis, which detects the point at which the amplified double stranded products separate into single strands during a heating process, directly relating to the length of the amplicon (Fig. 7). If dissociation takes place at multiple temperatures, the researcher knows that incorrect

sequences may be amplifying and new primer pairs or conditions are needed to increase specificity. Overall, real time PCR has revolutionized the way we analyze DNA and RNA and has made it possible to rapidly detect and determine the amount of starting target template in a sample in a cost efficient manner relative to other analysis methods used today.

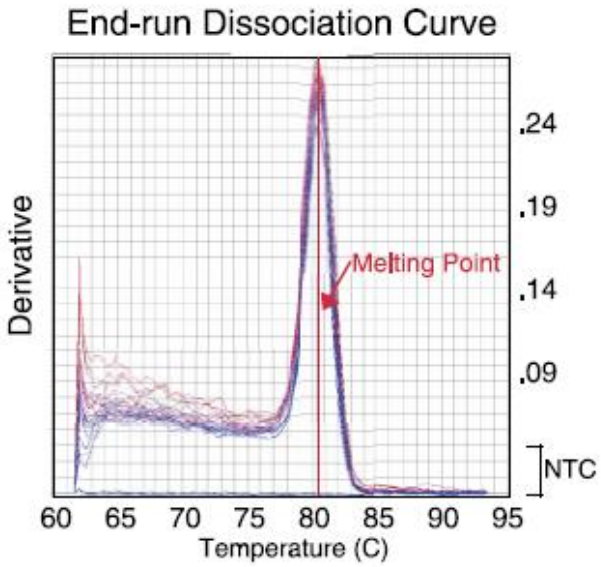

Figure 7. A typical melt curve analysis graph representation. This case shows one sharp peak, which suggests that a specific PCR product is being generated with the primers used. From Valasek and Repa 2005.

2. Objectives

2.1 Significance

The interface between the ocean and the atmosphere makes up roughly 70% of the planet's surface (Norkrans 1980). There is interest in surfactants found in the microlayer, as their accumulation can become visible in SAR satellite imagery in low wind speed conditions. This study will aid in our understanding of marine processes that affect oceanographic features seen by satellite. By using the microlayer sampling method

first described by Kurata (2012) and Vella (2012), this study looks to improve on the technique to further validate its use, which may aid in the formation of a standard sampling method that can be used by other researchers performing microlayer work. The use of this new method also has the potential to discover biological characteristics of the microlayer that are not yet known. Kurata (2012) and Vella's (2012) work was a pioneer project looking at differences in marine bacteria in slick and non slick conditions. However, their case study focused on only one region of the ocean (Straits of Florida). This study aims to use a different DNA analysis technique to compare *Bacillus* abundance in the Straits of Florida between different years as well as expand to another location of the world's ocean. The Gulf of Mexico was chosen as the second location due to the presence of natural oil seeps in this area, which change microbial activity and may affect *Bacillus* abundance in the near surface layer of the ocean. This project will further this field of study and contribute data so that other researchers can continue researching this understudied environment.

2.2 Hypotheses

1. There are differences in surfactant associated bacteria (*Bacillus*) relative abundance between the sea surface microlayer and underlying subsurface waters in slick and non slick conditions.
2. The relative abundance of surfactant associated genus *Bacillus* between the microlayer and the water column differs when comparing samples taken in the Straits of Florida and the Gulf of Mexico.
3. Bacteria located in subsurface waters play a role in the amount of surfactants at the sea surface, which may aid in the formation of natural sea slicks.

The objectives of this study are to:

1. Further advance DNA microlayer sampling techniques.
2. Introduce the use of real time PCR for the identification of microbes found in the microlayer and subsurface waters of the ocean.
3. Compare real time PCR findings to next generation 454 sequencing results from a case study performed in 2010.
4. Identify possible contamination from the air and lab using control membrane filters and real time PCR techniques.
5. Compare the relative abundance of the potential surfactant associated genus *Bacillus* in the sea surface microlayer and the subsurface water using samples collected in the Straits of Florida and Gulf of Mexico.

3. Materials and Methods

3.1 Sampling locations

For this study, microbial samples were collected in the Straits of Florida in September of 2013 in both slick and non slick conditions to compare the relative abundance of certain surfactant associated bacteria known from literature (Fig. 8). When possible, sampling was performed during SAR satellite overpasses, enabling our team to obtain images of slicks that were sampled and linking these bacteria to features seen in SAR imagery. Samples were also collected in the Gulf of Mexico in December 2013 (Fig. 8) as part of the CARTHE SCOPE research project taking place at Fort Walton

Beach in Destin, FL. These samples were also taken to compare abundance levels of certain bacteria in different regions of the ocean.

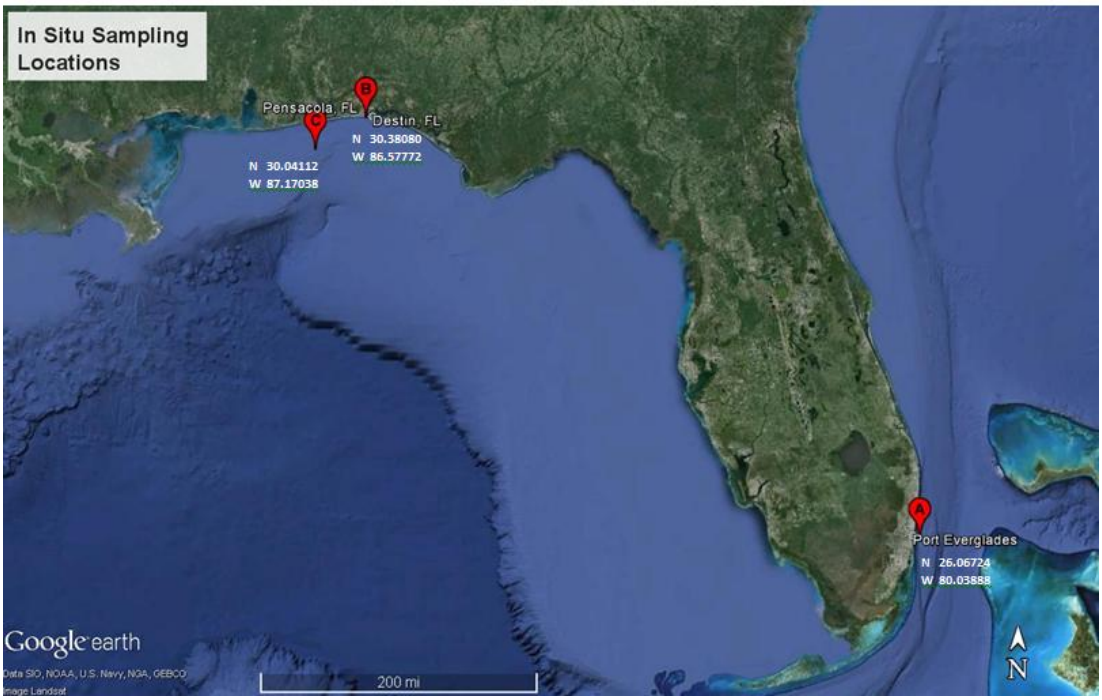

Figure 8. Sample collection sites. a) Straits of Florida (September 2013). b) Near shore of Destin, FL (December 2013). c) Approximately 20 miles offshore of Pensacola, FL (December 2013).

3.2 Microlayer sampling method

Microbial samples were collected from the sea surface microlayer using Isopore hydrophilic polycarbonate membrane filters measuring 47 mm in diameter with a 0.2 µm pore size (EMD Millipore). The polycarbonate film has a smooth glass-like surface, where any particles are retained once they come into contact with the filter. Prior to sampling, filters were taken directly from original packaging using sterile forceps and connected to a sterile hook and short fishing line within a biological safety cabinet. A loop was created at the opposite end of each short line, which is later used to connect the line to a fishing rod during microlayer sampling. The filter/hook combination was then

placed into a sterile bag and immediately sealed to reduce air contamination. Each filter bag was labeled using a different sample number, enabling our team to distinguish the filters when recording date, time, location, and weather conditions for each sample taken in the field. Sampling occurred on a small research boat, from the bow and away from the ship wake (sources of contamination and disturbance), as seen in Figure 9.

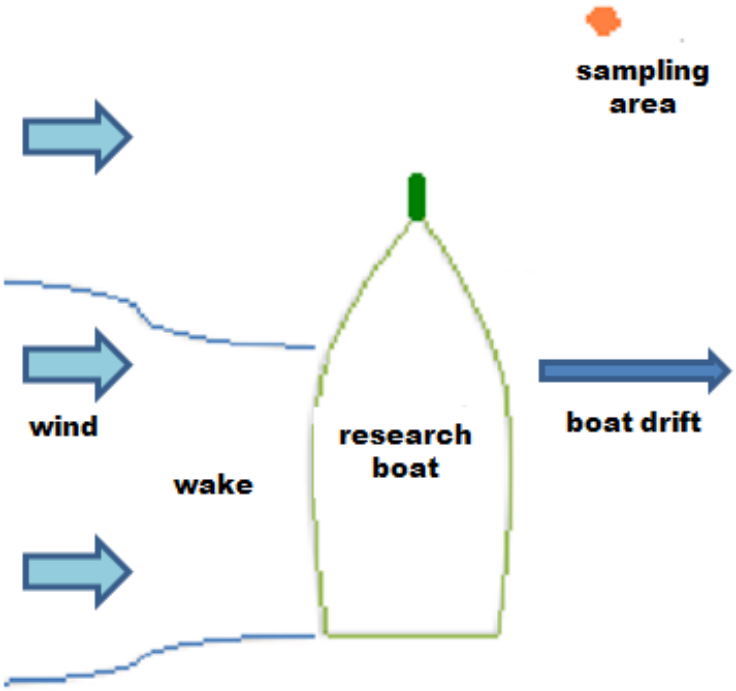

Figure 9. Ideal sea surface microlayer sampling area aboard research vessel, away from contamination and disturbance. Modified from Kurata 2012.

Once at the sampling site, the line attached to the filter and hook was connected to a 10 ft lightweight fishing rod and the filter was carefully pulled out of the bag without any contact from the researcher. The fishing rod enables the researcher to extend sampling away from the research boat and wake, which can easily disturb and contaminate the microlayer. The filter was placed directly on the sea surface for three to five seconds, ensuring that the filter did not become submerged. Microbial life found at

the sea surface sticks to the membrane filter due to surface tension, as seen in Figure 10. The filter was then lifted using the fishing rod and brought back to the boat, where it was collected using sterile tools and placed back in its original bag. The bag was then stored in a cooler containing dry ice to preserve the sample until it was placed in a -80°C freezer in the lab after fieldwork was completed.

Sampling procedures were recorded on video to make sure each sample only came into contact with the microlayer. Any filters suspected of coming into contact with anything other than the microlayer, sterile retrieval equipment, or the sterile bags were noted as such. Four "good" samples free of contamination and disturbance were taken at each site in order to have a large enough sample size for analysis while still having extra filters if problems arose during lab work. The method described above was originally designed by Kurata et al. (2012) to reduce contamination issues that plague other common microbial sampling techniques used today.

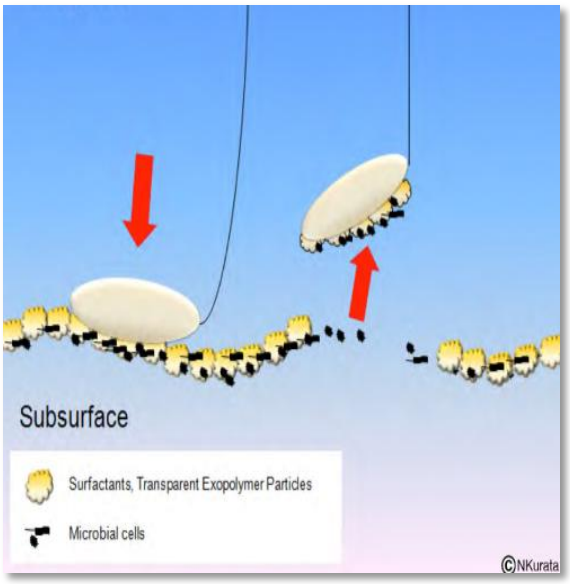

Figure 10. A membrane filter is placed directly on the sea surface, picking up surface materials and microbial cells before retrieval. From Kurata et al. 2012.

In sampling conditions involving strong wind, it can be very difficult to retrieve the filter from the fishing rod using sterile forceps, often doubling to tripling the amount of time needed to collect a good sample free of contamination. An improvement was successfully tested to provide a more calm area at the bow of the vessel in order to retrieve samples. This involves the use of multiple umbrellas attached to the boat railing to block winds coming from a particular direction, as seen in Figure 11. The improvement allowed us to sample efficiently in less than favorable conditions on a number of occasions and is recommended for use in future microlayer studies.

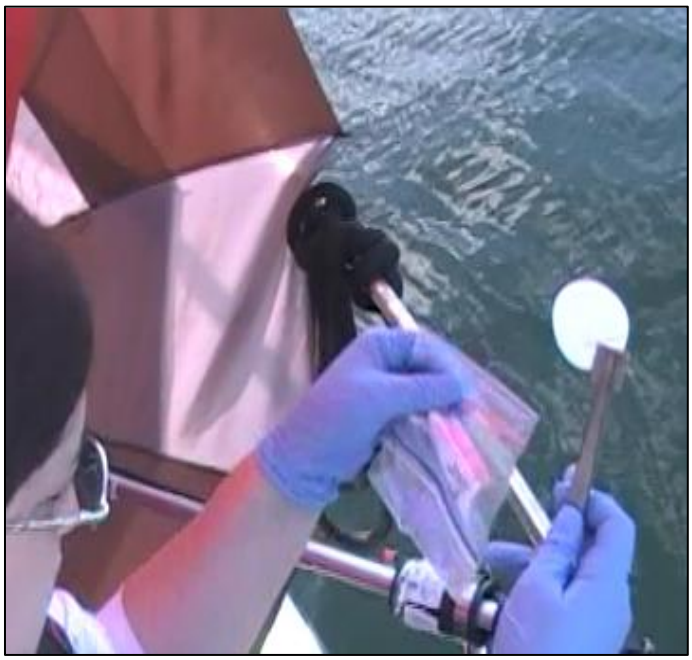

Figure 11. Filter collection after surface deployment has occurred. Filters are handled using sterile forceps and placed in a sterile bag within an area of decreased wind.

3.3 Subsurface sampling method

Subsurface samples were also taken in the same location that microlayer sampling took place to get a relative comparison between the bacterial communities found at

different water depths. An environmental peristaltic water pump (Barnant) was used to pump water from depth into a sterile bag without touching any surface except tubing that was sterilized with alcohol directly before sampling took place. A fishing rod was used to extend the sampling location away from the boat and wake. Tubing was directly attached to the pole, which had a flotation device at the end to ensure correct sampling depth. For this study, the sampling depth for subsurface water was 0.2 m (Fig. 12). Once the water was collected, a membrane filter was placed in the bag using sterile tools for five to ten seconds to pick up any microbial life. It was then retrieved and placed in a labeled bag, where it was stored in a similar manner to microlayer samples. Four good samples free of contamination from the boat and researcher were collected in each type of environment for comparisons.

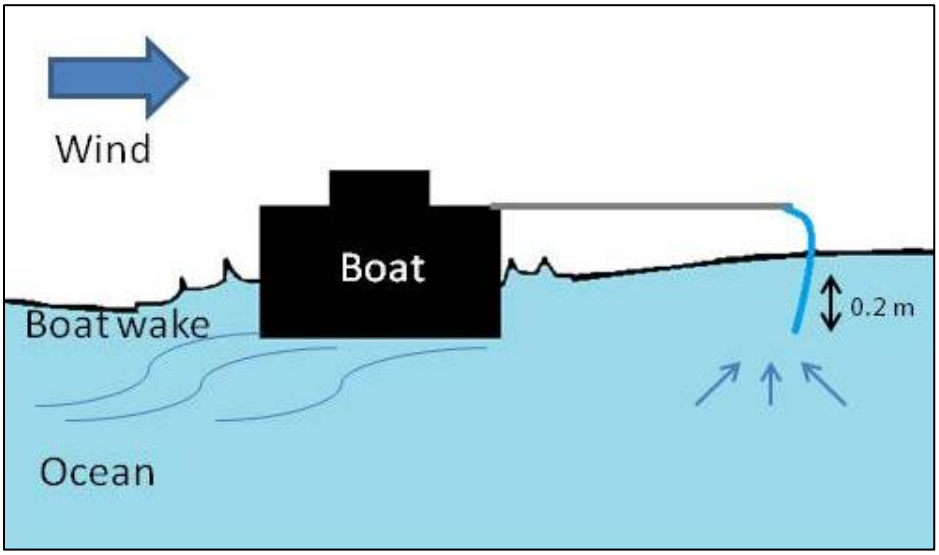

Figure 12. Subsurface sampling location away from the boat and boat wake. From Hamilton et al. 2015.

3.4 Controls

Similarly to Kurata et al. (2012), membrane filters were exposed to the air only at certain sampling locations to pick up any airborne microbial life. A number of filters

were also taken directly from their original packaging and placed in a sterile bag for analysis. These filters served as controls, giving our team an idea of what possible contaminants are residing in the air and lab setting. Knowing these contaminants, we can then exclude them from our results after analysis takes place.

3.5 SAR overpass

Satellite image acquisitions were planned prior to fieldwork from both the TerraSAR-X and RADARSAT-2 satellites to see any visible surface features at the sampling locations. A SAR image was ordered for TerraSAR-X satellite overpass of the Straits of Florida on September 15th, 2013 at 11:25 UTC (Fig 13). Microlayer and subsurface microbial sampling occurred during this overpass to link the microbial life found on the filters to features seen in satellite imagery. Unfortunately, due to the weather conditions of that day, there were no slicks present in the sampling area and no distinguishable features were seen in the SAR image.

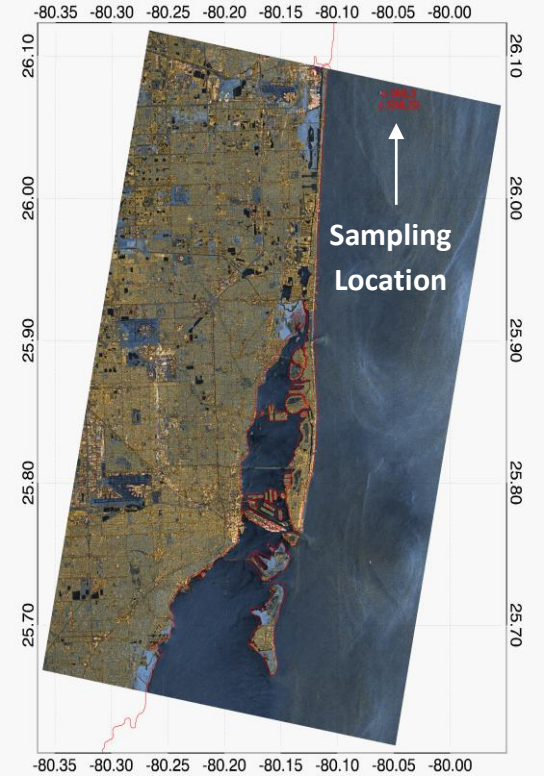

Figure 13. TerraSAR-X image of Straits of Florida acquired on 15th September, 2013 at 11:25 UTC in StripMap VV polarization mode 3 m resolution. The color coding is done according to image statistics including. e.g., intensity, gradient and entropy. It was chosen such that objects with strong local gradients get a high intensity (like in cities) and some natural colors remain of objects, e.g. water is blue.

During the 2013 sampling event in the Gulf of Mexico, image acquisition was planned for the 15th of December from RADARSAT-2 satellite overpass. The image was taken at 18:20 UTC (Fig 14). Due to less than ideal weather conditions, sampling did not take place on the day of satellite image acquisition. There were also no slick features seen from this image.

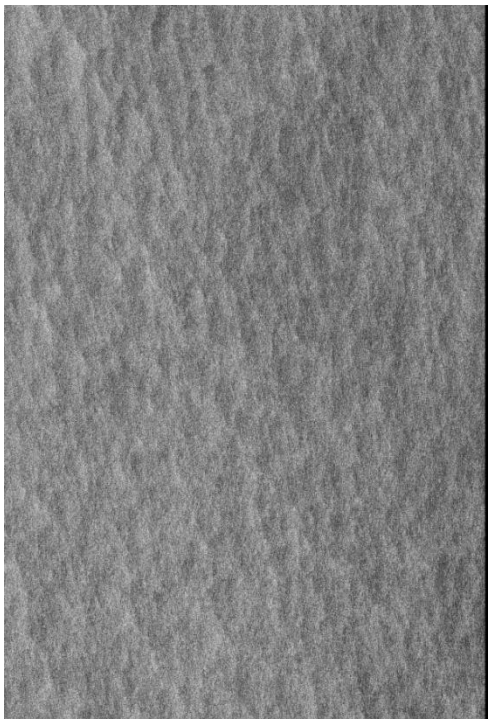

Figure 14. RADARSAT 2 image of Gulf of Mexico sampling location aquired on 17th December, 2013.

3.6 DNA lab analysis

DNA Extraction

Membrane filters collected during fieldwork were brought to a lab in NSU's Farquhar Arts and Sciences Parker building for DNA extraction using the QIAamp DNA Investigator Kit (QIAGEN). The kit protocol for extracting from paper was followed. This process involves each filter being cut into pieces using sterile equipment and running the pieces through a series of buffer solutions to detach microbes from the filter and cause cell lysis, which releases DNA into a solution that is used for genus identification at a later time. Large particles and other materials that may affect analysis at a later stage are filtered out during this process, leaving only clean DNA fragments from each environment sampled. Tests using nested PCR (1st primer pair – 27F (5'-

AGAGTTTGATCMTGGCTC AG-3') and 1100R (5'-GGGTTGCGCTCGTTG-3'), 2nd primer pair – 357F (5'-CTCCTACGGGAGGCAGCAG-3') and 1100R (5'-GGGTTGCGCTCGTTG-3')) on a subset of samples were performed to ensure the extraction method described was successful in collecting DNA fragments from the filters (Lane 1991). The amplified products were observed visually using the agarose gel electrophoresis technique.

PCR

The Polymerase Chain Reaction (PCR) technique was used to amplify DNA fragments belonging to types of bacteria found in each sample. PCR is vital to many microbial studies that involve a small of amount of DNA present in samples. To begin this process, primers that are designed to attach to specific regions of a gene found in a certain organism are added to a DNA sample along with a number of other necessary materials. In this case, a fragment of 16s rRNA gene found in all bacteria was first targeted for amplification using universal primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Galkiewics and Kellogg 2008). The first PCR stage was used to combat the small initial quantity found in each extracted sample. This step is considered the beginning of a nested PCR, as the resulting amplicons were then used for a real time PCR run. The materials required for amplification consisted of 1 µl of extracted DNA sample, 1 µl of 10 µM 8F primer, 1 µl of 10 µM 1492R primer, 2.5 µl 10X buffer solution, 0.5 µl dNTP, 0.2 µl *Taq* polymerase, and 19 µl H_20 per sample. The buffer solution, dNTP, and *Taq* polymerase were from a *Taq* PCR core kit (QIAGEN). The PCR machine used for this study was a BIO RAD MyCycler thermal cycler. The initial PCR run consisted of 95°C

for 2 min, followed by 15 cycles of 95°C for 30 sec, 45°C for 30 sec, and 72°C for 1 min, and an elongation period at 72°C for 5 min. Following the PCR run, the products were stored in a freezer until their use in the final real time PCR reaction.

Real Time PCR

Real time PCR enables researchers to determine the relative abundance of a certain organism or group of organisms that are present in a sample through the ability to quantify the amount of amplification taking place each cycle (Pace 2009). For this study, a dye called SYBR green I was used to make this process possible. SYBR green I is added to the DNA solution and attaches to double stranded DNA, emitting fluorescence when it does so. The PCR machine reads fluorescence levels every cycle, so if the target is present, there will be a doubling effect of amplicons and fluorescence amounts detected. Relative abundance level comparisons can then be made between each sample.

For this study, the primer pair 265F (5'-GGCTCACCAAGGCAACGAT-3') and 525R (5'-GGCTGCTGGCACGTAGTTAG-3') designed to amplify 16s gene sequences specific to the genus *Bacillus* were acquired (Xiao et al. 2011). A positive culture of *Bacillus* was obtained, along with a culture of *Acinetobacter*, *Pseudomonas*, and *Corynebacterium*. These cultures served to verify that the acquired primers were specific to *Bacillus*.

The materials required for amplification consisted of 2 µl of PCR amplicon sample, 1 µl of 10µM 265F primer, 1 µl of 10µM 525R primer, 15 µl SYBR green I master mix solution (Applied Biosystems), and 9 µl H_20 per sample. The StepOne Real-Time PCR System (Applied Biosystems) was used to amplify the targeted gene

sequences and each run consisted of 95°C for 15 min, followed by 25 cycles of 94°C for 30 sec, 62°C for 30 sec, and 72°C for 2 min. This was followed by an elongation period at 72°C for 10 min and a melt curve stage to determine amplicon lengths.

3.7 Data analysis

Real time PCR data was analyzed using the StepOne Software included with StepOne and StepOne Plus PCR systems. This software package enables the researcher to set a number of parameters for each PCR run, making it easy to customize a run to a researcher's preference. Following the PCR run, the software automatically flags any samples with abnormal amplification patterns for further review. StepOne Software also uses Melt Curve Analysis to determine if the amplicons being amplified are all similar in length. This process involves heating every sample and determining when the sequence strands split in two. The longer the strand, the longer it takes to break the strand apart. This analysis tool provides a form of validation that the primer pair is working correctly and not attaching to the wrong areas of the DNA strand.

Comparisons between samples were based on the cycle number when each sample crossed a certain threshold level of fluorescence detected (directly relating to the amount of amplicons present in the sample). The automatic threshold for each PCR run is determined by the software using a set of algorithms. The software also enables the user to change this threshold level manually to better fit the graph presented, as it is important that the samples are compared during the linear phases of amplification. The real time PCR machine and software package described above enables the rapid analysis of a large number of samples at once (up to 48 samples per run in this case). Abundance differences

were presented in a bar graph format using the average of 4 abundance levels from samples taken in the same location. Abundance = $2^{(28-ct)}$ where ct is the cycle number when a sample crosses the set threshold. This formula was created to illustrate the differences in each sample using the CT value from the real time PCR run. 70% Confidence intervals using a t-distribution test were then applied to each bar.

4. Results

4.1 Successful extraction test and *Bacillus* primer specificity

A subset of DNA samples extracted from the filters were amplified using the PCR methods described in Section 3.6 to ensure that the extraction process yielded a sufficient amount of DNA. Gel electrophoresis results using the amplified PCR products are shown in Figure 15. Bands were present for all samples tested, confirming a successful extraction process.

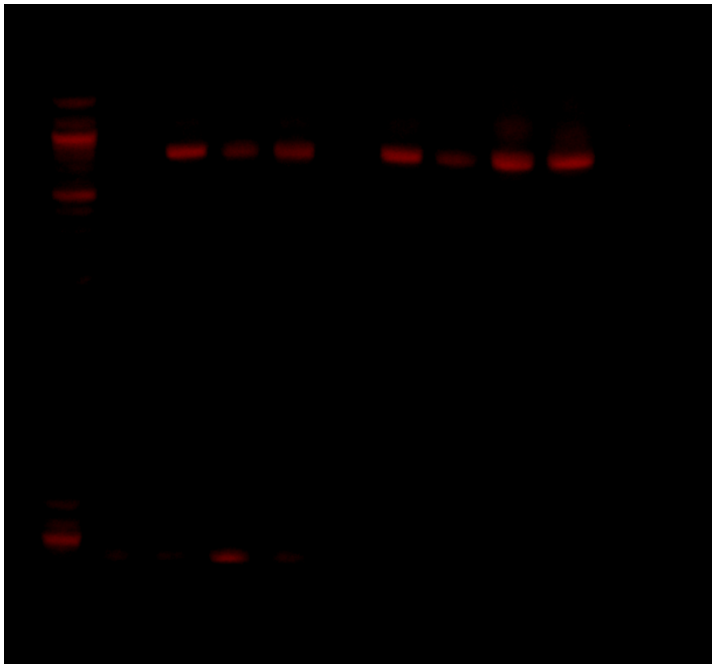

Figure 15. Results of agarose gel electrophoresis to confirm DNA extraction success. 12 samples were tested, along with a negative control (1st row, well 2).

Using cultures of bacteria obtained from Carolina Biological Supply Company (http://www.carolina.com/living-organisms/prokaryotes/bacteria/10631.ct), the real time PCR test to ensure the Bacillus specific primers work correctly (described in Section 3.6) showed that the positive Bacillus control was the only sample to cross the set threshold. All other bacteria cultures showed insignificant amplification within the cycle count used (Fig. 16). A positive Bacillus control as well as a melt curve analysis was included for all real time PCR reactions to give more confidence on the correct amplicon length.

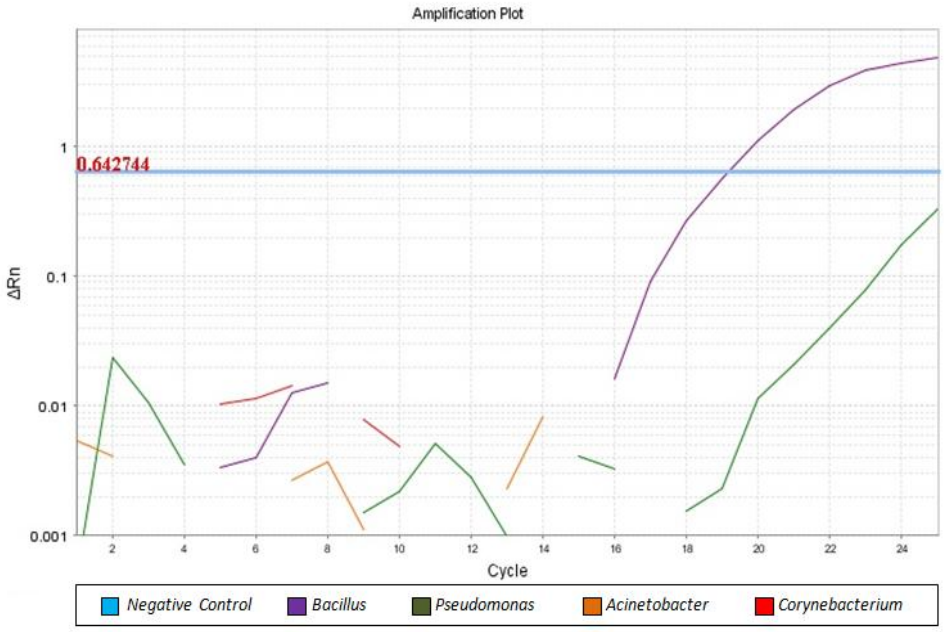

Figure 16. Bacillus specific primer real time PCR test using cultures of *Bacillus*, *Pseudomonas*, *Acinetobacter*, and *Corynebacterium*. *Bacillus* was the only sample to cross the set threshold for the real time PCR run.

4.2 Real time PCR vs. 454 sequencing results

Using our real time PCR method on the 4 samples from Kurata's (2012) study previously described (Figure 17), we determined that *Bacillus* was much more abundant

in the slick subsurface sample than the other 3 samples analyzed. It was also determined that running the initial PCR for too many cycles (30+) before the nested real time PCR stage caused a reduction in difference between the 4 samples. We found that reducing the initial stage from 30 cycles to 15 cycles kept the relative abundance differences intact, which closely resembled the 454 sequencing results obtained by Kurata (2012) (Fig. 18). This result solidified the decision to use real time PCR and the *Bacillus* specific primers obtained for the remainder of the samples collected in 2013.

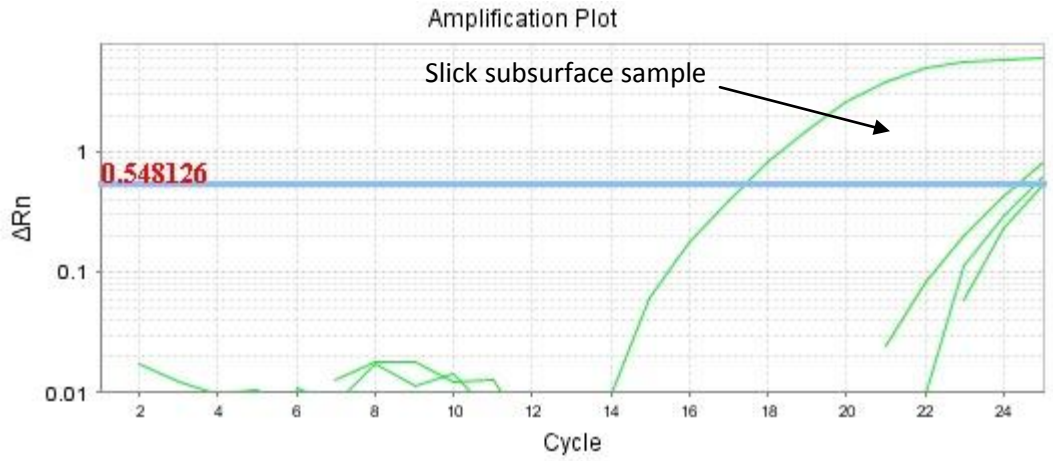

Figure 17. Real time PCR results of *Bacillus* abundance found in four samples from Kurata's (2012) study. The highest abundance was found to be in the slick subsurface sample, which agrees with the result that Kurata found using 454 sequencing.

a) 454 sequencing b) Real time PCR

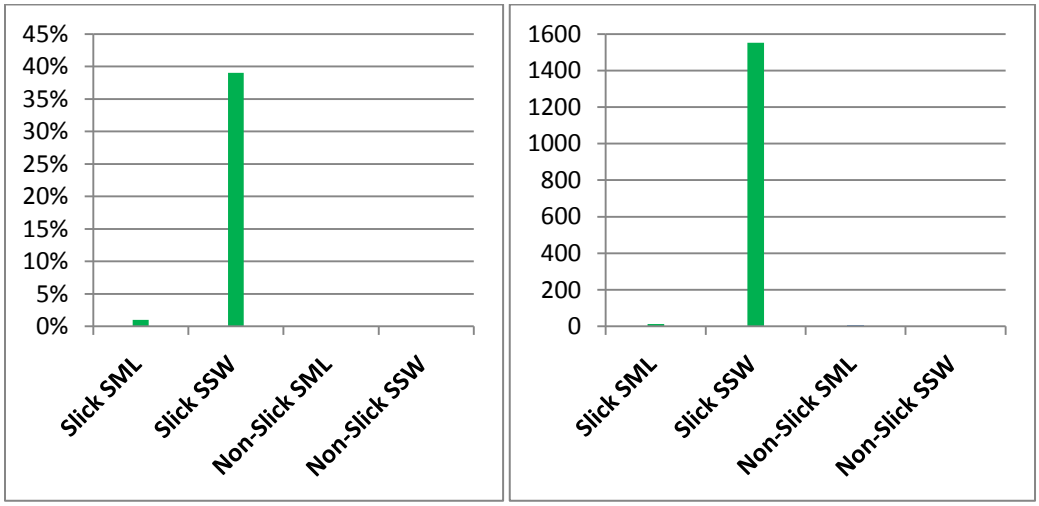

Figure 18. Abundance levels of the surfactant associated genus *Bacillus* from samples collected during the 2010 case study in the Straits of Florida. a) Results using 454 sequencing techniques. The abundance of *Bacillus* genus identified on each sample relative to all bacterial genera; b) Results using real time PCR techniques. Real time PCR relative abundance=$2^{(28-ct)}$. Ct value = cycle count when the amount of amplification reaches a determined threshold level for each sample during quantitative PCR run. (SML stands for sea surface microlayer, SSW, for subsurface water.)

4.3 Analysis of 2013 Samples

A total of 56 samples (excluding controls) were analyzed using the techniques described above. For the Straits of Florida study, 24 samples were compared within the same real time PCR run. 32 Samples collected in the Gulf of Mexico were analyzed using a separate real time PCR run, as the machine could only hold up to 48 samples at a time. Although the methods leading to the results of each group of samples were the same, the groups should not be directly compared, as they did not come from the same master mix solutions and were extracted and analyzed on a different day.

4.3.1 Straits of Florida

Although the relative abundance varied considerably with each sample, the targeted genus was present in all samples analyzed from this geographical location (Fig. 19) .Of the 24 samples analyzed from the Straits of Florida, 16 were from September 14th

and 8 were collected September 15th of 2013 (Table 1). Slick conditions were found September 14th while on the research boat and 4 good samples were collected from both the sea surface microlayer and the subsurface water. 4 Samples from each type of environment were also collected in nearby non slick conditions for comparisons.

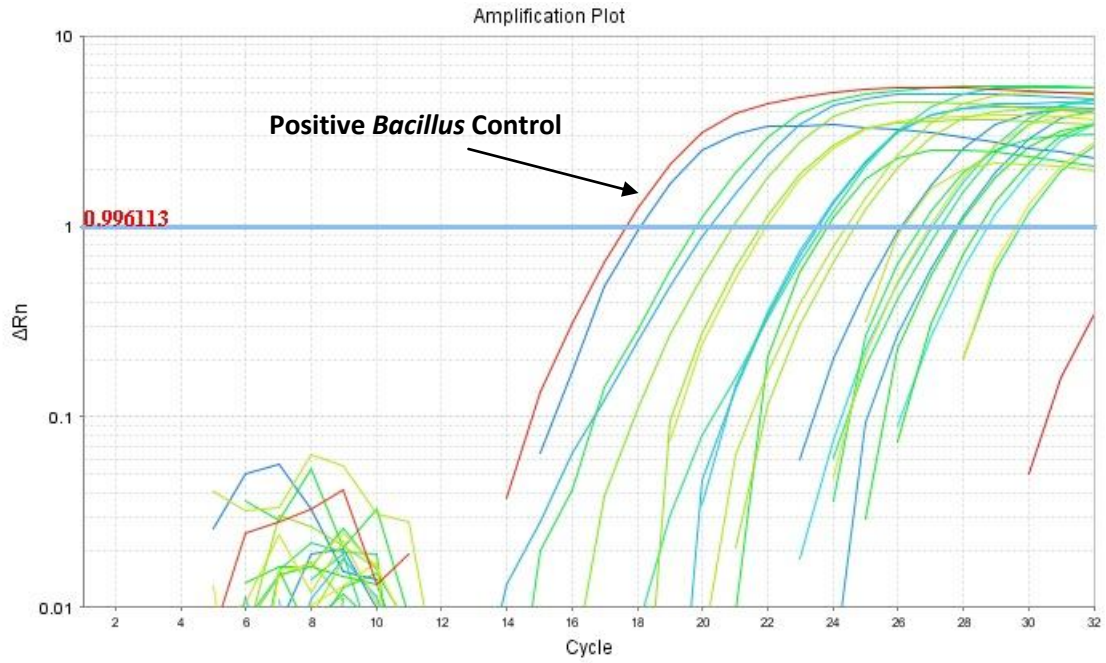

Figure 19. Real time PCR results showing amplification taking place in all samples and the positive control. The amount of fluorescence emitted from each sample (y axis) directly relates to the amount of amplification taking place. The software included with this system chooses a set threshold (in this case 0.996113) based on the linear phase of amplification for each line (corresponding to a sample). Relative abundance is compared using the cycle number at which each line crosses the threshold level. No significant amplification occurred for the air filter and negative control.

Table 1. Samples collected from the Straits of Florida on September 14th and September 15th of 2013. Conditions were recorded during each sampling event and are shown below. The CT Value represents the cycle number at which each sample crossed a set threshold level during the real time PCR run.

DATE	NUMBER	SLICK/NO SLICK	WIND	WAVES	LOCATION	TIME (UTC)	CT VALUE
9/14/2013	SML 5	Slick	0-2 m/s	1/2 ft	N 26.04400 W 80.01973	13:55	21.7
9/14/2013	SML 7	Slick	0-2 m/s	1/2 ft	N 26.04575 W 80.01973	14:03	20.9
9/14/2013	SML 11	Slick	0-2 m/s	1/2 ft	N 26.04842 W 80.02038	14:18	24.4
9/14/2013	SML 16	Slick	0-2 m/s	1/2 ft	N 26.05014 W 80.02039	14:29	24.6
9/14/2013	SSW 10	Slick	0-2 m/s	1/2 ft	N 26.05269 W 80.02117	14:46	26.9
9/14/2013	SSW 7	Slick	0-2 m/s	1/2 ft	N 26.05269 W 80.02117	14:46	29.5
9/14/2013	SSW 8	Slick	0-2 m/s	1/2 ft	N 26.05269 W 80.02117	14:46	26.1
9/14/2013	SSW 6	Slick	0-2 m/s	1/2 ft	N 26.05269 W 80.02117	14:46	21.9
9/14/2013	SML 18	No Slick	0-2 m/s	1/2 ft	N 26.06239 W 80.01761	15:18	28.4
9/14/2013	SML 19	No Slick	0-2 m/s	1/2 ft	N 26.06293 W 80.01789	15:22	23.8
9/14/2013	SML 20	No Slick	0-2 m/s	1/2 ft	N 26.06293 W 80.01789	15:23	19.8
9/14/2013	SML 21	No Slick	0-2 m/s	1/2 ft	N 26.06346 W 80.01817	15:25	27.8
9/14/2013	SSW 4	No Slick	0-2 m/s	1/2 ft	N 26.06521 W 80.01877	15:33	23.7
9/14/2013	SSW 3	No Slick	0-2 m/s	1/2 ft	N 26.06521 W 80.01877	15:33	26.6
9/14/2013	SSW 5	No Slick	0-2 m/s	1/2 ft	N 26.06521 W 80.01877	15:33	29.7
9/14/2013	SSW 9	No Slick	0-2 m/s	1/2 ft	N 26.06521 W 80.01877	15:33	27.2
9/15/2013	SML 3	No Slick	3-4 m/s	2-4 ft	N 26.04258 W 80.03654	11:39	23.4
9/15/2013	SML 29	No Slick	3-4 m/s	2-4 ft	N 26.03771 W 80.03797	11:55	23.5
9/15/2013	SML 30	No Slick	3-4 m/s	2-4 ft	N 26.03695 W 80.03824	11:58	28.7
9/15/2013	SML 32	No Slick	3-4 m/s	2-4 ft	N 26.03503 W 80.03898	12:04	27.0
9/15/2013	SSW 11	No Slick	3-4 m/s	2-4 ft	N 26.05029 W 80.03475	11:15	26.0
9/15/2013	SSW 12	No Slick	3-4 m/s	2-4 ft	N 26.05029 W 80.03475	11:15	18.0
9/15/2013	SSW 13	No Slick	3-4 m/s	2-4 ft	N 26.05029 W 80.03475	11:15	27.7
9/15/2013	SSW 2	No Slick	3-4 m/s	2-4 ft	N 26.05029 W 80.03475	11:15	20.2

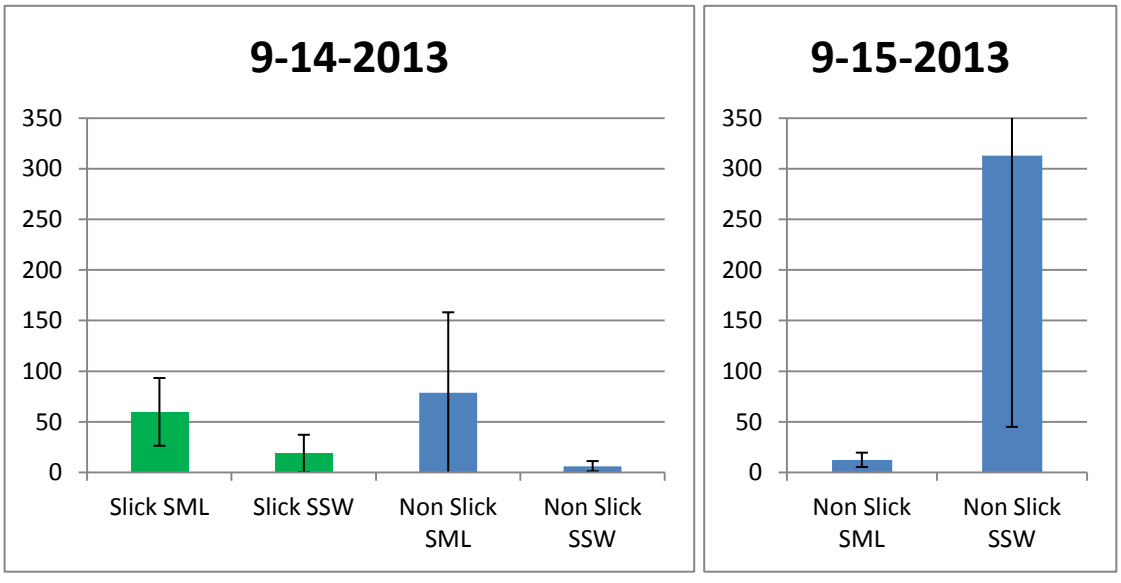

Figure 20. Relative abundance of *Bacillus* in samples collected in the Straits of Florida 2013 shown with 70% confidence intervals using t-distribution. The relative abundance shown is based on the average of 4 samples taken in the same location. Abundance = $2^{(28-ct)}$ where ct is the cycle number when a sample

crosses the set threshold. a) Samples collected on September 14th, 2013. b) Samples collected on September 15th, 2013.

Figure 20 shows the relative abundance comparisons of *Bacillus* for the samples collected on September 14th and 15th, 2013. The graph above is based on the CT value for each sample described previously. To better illustrate the relative abundance differences, the formula $2^{(28-ct)}$ was used where ct is the cycle number when a sample crosses the set threshold. Each group of 4 samples that were collected in the same area were averaged using the formula above and each average is shown in the graph (Fig. 20). In slick conditions, *Bacillus* was shown to be indistinguishable in relative abundance between the sea surface microlayer and the subsurface water directly below because of relatively small statistics and, as a result, large confidence intervals. The results were similar at the non slick site, as *Bacillus* abundance differences were not possible to distinguish with the 70% confidence intervals.

In the non slick area sampled on September 15th, *Bacillus* was more abundant in the subsurface water than in the samples taken from the sea surface microlayer and was resolvable using 70% confidence intervals. Of all of the samples collected and analyzed from the Straits of Florida during the two sampling trips, the highest amount of *Bacillus* was found in this non slick subsurface area.

4.3.2 Gulf of Mexico

Of the 32 samples collected and analyzed from the Gulf of Mexico 2013 trip, 8 samples were compared from December 11th, while 16 were compared from December 16th (Fig. 21, Table 2). The remaining 8 of the PCR run were collected on December 17th about 20 miles offshore of the Pensacola, FL area. Slicks were visually identified and

collected from on December 11th and 16th along with non slick samples for comparisons. Unfortunately, there were no slicks present in our general area on the 17th of December.

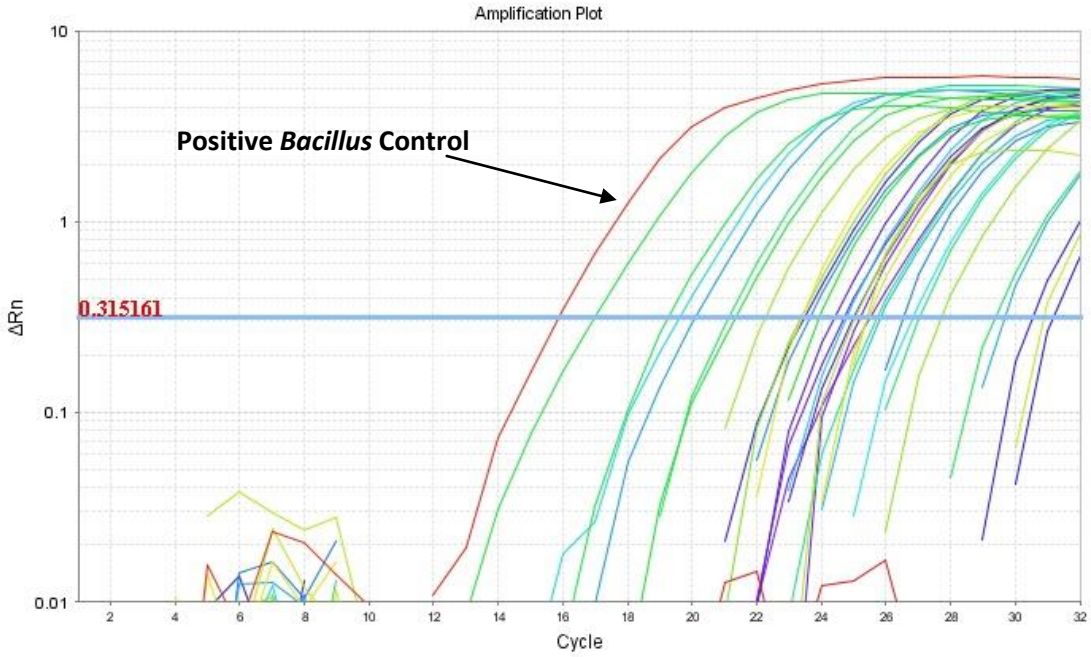

Figure 21. Real time PCR results showing amplification taking place in all samples and the positive control. The amount of fluorescence emitted from each sample (y axis) directly relates to the amount of amplification taking place. Relative abundance is compared using the cycle number at which each line crosses the threshold level.

Table 2. Samples collected from the Gulf of Mexico on December 11th, 16th, and 17th of 2013. Sampling conditions were recorded for each and are shown below along with the CT Value obtained from the real time PCR run.

DATE	NUMBER	SLICK/NO SLICK	WIND	WAVES	LOCATION	TIME (UTC)	CT VALUE
12/11/2013	SML 83	Slick	1.5 m/s	1/2 ft	N 30.38043 W 86.57733	17.58	25.3
12/11/2013	SML 84	Slick	1.5 m/s	1/2 ft	N 30.38080 W 86.57772	18:00	23.2
12/11/2013	SML 85	Slick	1.5 m/s	1/2 ft	N 30.38094 W 86.57778	18:03	30.7
12/11/2013	SML 89	Slick	1.5 m/s	1/2 ft	N 30.38110 W 86.57832	18.06	25.0
12/11/2013	SSW 57	Slick	1.5 m/s	1/2 ft	N 30.38201 W 86.57957	18:11	22.1
12/11/2013	SSW 31	Slick	1.5 m/s	1/2 ft	N 30.38201 W 86.57957	18:11	25.0
12/11/2013	SSW 29	Slick	1.5 m/s	1/2 ft	N 30.38201 W 86.57957	18.11	27.6
12/11/2013	SSW 64	Slick	1.5 m/s	1/2 ft	N 30.38201 W 86.57957	18.11	23.3
12/16/2013	SML 116	Slick	<3 m/s	<1 ft	N 30.36051 W 86.60643	22:20	17.1
12/16/2013	SML 109	Slick	<3 m/s	<1 ft	N 30.36077 W 86.60622	22:22	23.8
12/16/2013	SML 110	Slick	<3 m/s	<1 ft	N 30.36104 W 86.60610	22:24	19.2
12/16/2013	SML 112	Slick	<3 m/s	<1 ft	N 30.36130 W 86.60587	22:26	21.3
12/16/2013	SSW 56	Slick	<3 m/s	<1 ft	N 30.36293 W 86.60555	22:34	29.3
12/16/2013	SSW 58	Slick	<3 m/s	<1 ft	N 30.36293 W 86.60555	22:34	21.0
12/16/2013	SSW 59	Slick	<3 m/s	<1 ft	N 30.36293 W 86.60555	22:34	26.9
12/16/2013	SSW 60	Slick	<3 m/s	<1 ft	N 30.36293 W 86.60555	22:34	25.7
12/16/2013	SML 113	No Slick	<3 m/s	<1 ft	N 30.36500 W 86.60882	22:51	29.4
12/16/2013	SML 114	No Slick	<3 m/s	<1 ft	N 30.36505 W 86.60867	22:52	20.1
12/16/2013	SML 118	No Slick	<3 m/s	<1 ft	N 30.36508 W 86.60865	22:53	23.5
12/16/2013	SML 117	No Slick	<3 m/s	<1 ft	N 30.36528 W 86.60862	22:55	26.3
12/16/2013	SSW 49	No Slick	<3 m/s	<1 ft	N 30.36456 W 86.60900	22:43	24.7
12/16/2013	SSW 46	No Slick	<3 m/s	<1 ft	N 30.36456 W 86.60900	22:43	19.6
12/16/2013	SSW 55	No Slick	<3 m/s	<1 ft	N 30.36456 W 86.60900	22:43	25.8
12/16/2013	SSW 45	No Slick	<3 m/s	<1 ft	N 30.36456 W 86.60900	22:43	26.7
12/17/2013	SML 159	No Slick	5 m/s	1 ft	N 29.57306 W 87.16007	12:40	30.4
12/17/2013	SML 158	No Slick	5 m/s	1 ft	N 29.57361 W 87.15983	12:43	31.0
12/17/2013	SML 156	No Slick	5 m/s	1 ft	N 29.57360 W 87.15935	12:47	25.0
12/17/2013	SML 155	No Slick	5 m/s	1 ft	N 29.57361 W 87.15900	12:49	23.4
12/17/2013	SSW 47	No Slick	5 m/s	1 ft	N 29.57373 W 87.15745	12:57	24.4
12/17/2013	SSW 125	No Slick	5 m/s	1 ft	N 29.57373 W 87.15745	12:57	24.7
12/17/2013	SSW 126	No Slick	5 m/s	1 ft	N 29.57373 W 87.15745	12:57	25.1
12/17/2013	SSW 127	No Slick	5 m/s	1 ft	N 29.57373 W 87.15745	12:57	25.6
12/17/2013	128	N/A	5 m/s	N/A	N 29.57373 W 87.15745	12:57	N/A

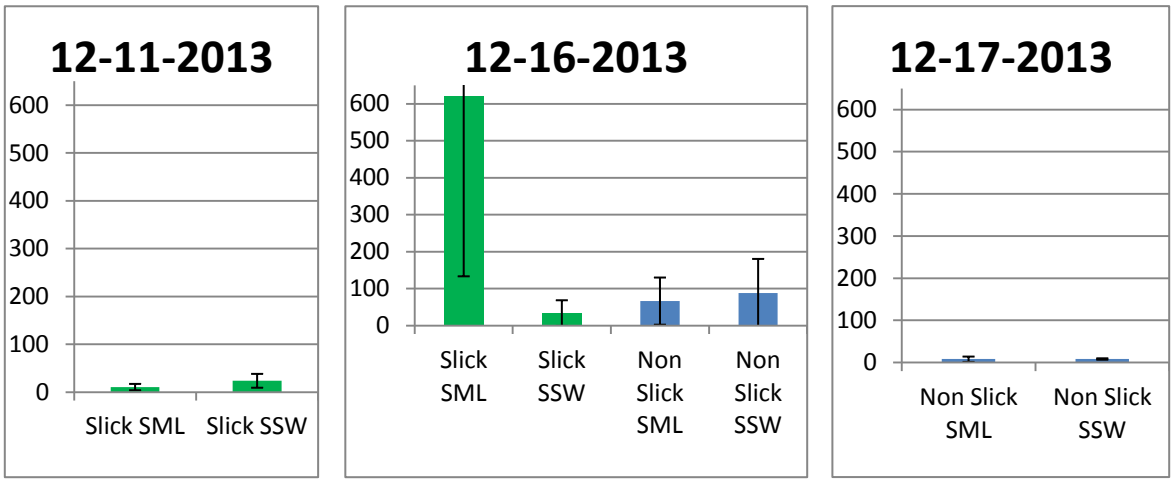

Figure 22. Relative abundance of *Bacillus* in samples collected in the Gulf of Mexico 2013 shown with 70% confidence intervals using t-distribution. The relative abundance shown is based on the average of 4 samples taken in the same location. Abundance = $2^{(28-ct)}$ where ct is the cycle number when a sample crosses the set threshold. a) Samples collected on December 11th, 2013. b) Samples collected on December 16th, 2013. c) Samples collected on December 17th, 2013.

In a similar manner to the samples analyzed from the Straits of Florida, Figure 22 shows relative abundance levels of *Bacillus* from the three days listed above. Each column represents the average relative abundance of the 4 samples collected in the listed area and follows the formula, Abundance = $2^{(28-ct)}$ where ct is the cycle number when a sample crosses the set threshold. Of the samples collected in slick conditions on the 11th of December, the differences in relative *Bacillus* abundance between the microlayer and the subsurface water could not be resolved within 70% confidence intervals. On December 16th, *Bacillus* was more abundant in the sea surface microlayer than the water column in slick conditions and also more abundant than both the microlayer and water column samples taken from non slick conditions taken that same day. This difference could be resolved using 70% confidence intervals. Samples taken much farther offshore on the 17th of December showed a low abundance of *Bacillus* bacteria, possibly pointing to this genus of bacteria thriving closer to the coast where more nutrients are present.

Similarly to the samples collected from the Straits of Florida, we detected a presence of *Bacillus* in all environmental samples taken. Relative abundance varied significantly between each sample and in some cases could be resolved within 70% confidence intervals. The highest amount of *Bacillus* detected was from a sample taken on December 16th, in slick microlayer conditions. This sampling site was located in the coastal area of Destin, FL (see Fig. 8).

4.3.3 Controls

Air and non exposed filters were analyzed in the same manner as the environmental samples described above. An air exposed filter taken in the Gulf of Mexico was extracted and used for analysis. During initial tests to determine the best cycle conditions to specify the detection of *Bacillus*, contamination was found on a non exposed filter and an air control filter, as amplification was taking place within the sample tube at a late stage of the real time PCR (Fig. 23). However, once cycle conditions were modified to match the original primer suggestions from Xiao et al. (2011), the air exposed filter (SML 128 in Tables 1 and 2) did not show any contamination. It is possible non specific amplification was taking place before these modifications or contamination took place while working in the lab. When working with such small quantities of DNA material from bacteria, it is vital to practice sterile techniques, as many types of microbes can be found in the lab setting.

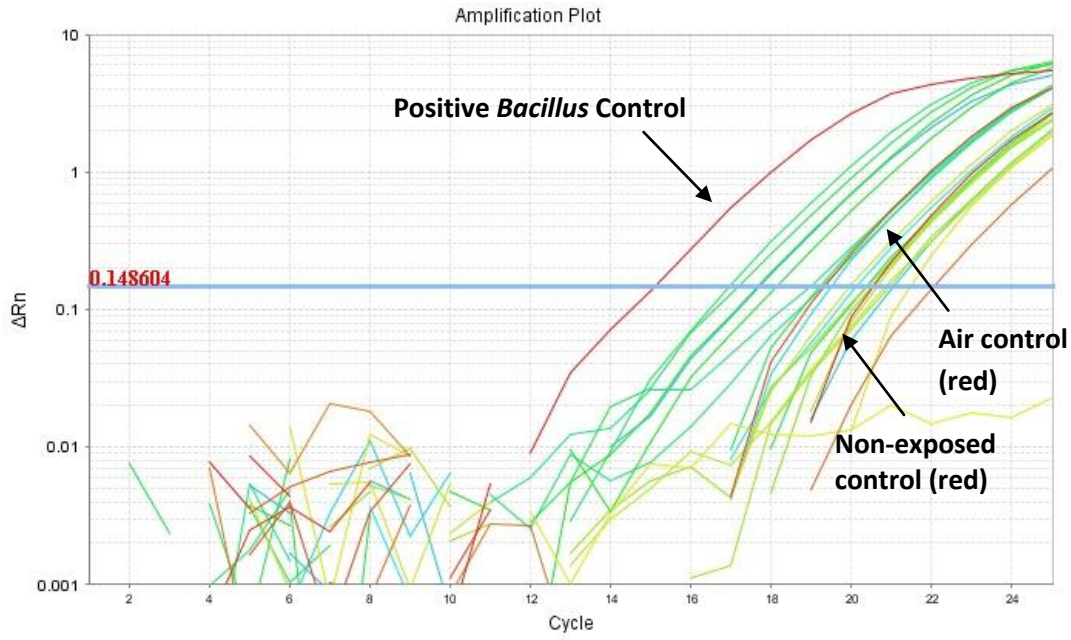

Figure 23. Example of *Bacillus* detection on air and non exposed filters. This real time PCR run was performed prior to modifications to increase specificity for the detection of *Bacillus*.

5. Discussion

5.1 Evaluation of microlayer sampling method

The microlayer sampling method initially developed by Kurata et al. (2012) and used in this study should be considered for future microlayer projects for a number of reasons. The method focuses on reducing contamination from many sources, including the research boat and wake that it produces. Other microlayer sampling methods used today (Section 1.5) do not address the need for the sampling area to be positioned away from the boat. Because the microlayer makes up only the uppermost 1 mm of the ocean, any wave motion from a structure such as a boat will almost certainly affect the thin layer and microbial flora within.

The method used in this study benefits from the fact that the material picking up microbial life is not being reused in any way. After each deployment, the filter only

comes into contact with water from the targeted sampling area and is immediately stored in a sterile bag. Methods such as the glass plate and metal screen reuse the device coming into contact with the microlayer many times. Even with vigorous cleaning between each sampling event, using the same device over and over increases the chance of contamination from a previous sample. The time between each sample collected and clean up time are also increased with the glass plate and metal or mesh screen method due to the need to clean each so often.

The filters used are estimated to sample the uppermost 50 μm of the ocean, which is a much thinner area than other techniques used (Cunliffe et al. 2009; Garrett 1965). Because the microlayer is thought to only make up the uppermost 1 mm and there is a distinct community present, this filter technique makes it more likely that the microbial communities beneath the sea surface microlayer are not being sampled as well.

The filter method used allows for most of the preparation to be done in the lab rather than on the research boat. Once out in the field, microlayer sample collection simply involves the quick attachment of the filter/hook combination to a swivel on a fishing rod. Samples can be collected quickly without the hassle of moving heavy or large equipment around on the boat, such as a rotating drum. No time is needed to clean any devices between filter deployments, which is a benefit if the researcher wants to sample an area before it changes significantly from wave action. Polycarbonate filters are used for a number of DNA studies other than those focused on the sea surface microlayer. Protocols for extracting DNA from these materials is common and makes it possible for consistency and direct comparisons between future studies.

The use of PCR and real time PCR allowed us to rapidly detect the presence of bacterial DNA on our collected filters. This method also enabled us to determine if bacteria were on air filter and non exposed filter controls. This made us aware of the potential contaminants in the air during fieldwork as well as contaminants in the lab setting during analysis. Although *Bacillus* was the main focus for this study, PCR makes it possible to detect a number of types of bacteria or other types of organisms in each sample. This requires other primer pairs created to amplify another target as well as enough initial DNA to run multiple PCRs. Many primer pairs have already been developed to detect sequences specific to bacteria and other organisms and they can be ordered. It is also possible to design primers to target a certain sequence of DNA, although this was not done for this study.

5.2 Straits of Florida Comparisons

There was no significant difference in *Bacillus* abundance between the sea surface microlayer and the water column from slick and non slick samples, at 70% confidence intervals, taken on September 14th, 2013 (Fig. 20). Samples taken the following day, September 15th, showed a higher abundance of *Bacillus* in the water column than in the sea surface microlayer. The reason for this difference in abundance compared to the previous day may have to do with the weather conditions at the air sea interface. On the 14th, surface conditions were very calm with minimal wind speed (under 2 m/s) and wave height (1/2 ft) (Table 1). Such calm surface conditions enabled the presence of slicks, which we were able to sample. The following day, conditions were very different with a higher wind speed (up to 4 m/s) and larger wave height (up to 1.2 m). These conditions most likely played a role in the lack of slicks found on that day. Rough sea surface

conditions would disturb the microlayer and the microbial communities found within. Microbes below the surface would be less disturbed, and this may help to explain the lack of *Bacillus* at the surface.

The first study using a similar microlayer sampling technique done in 2010 by Kurata (2012) focused on a very small sample size of 1 sample per area. Additionally, the filter (47 mm in diameter) only picked up a very small area when deployed. The abundance of certain microbes in a specific area can change very quickly due to oceanic processes. The samples from Kurata's (2012) study are also a special case due to the Deepwater Horizon Oil Spill event that was taking place in the Gulf of Mexico at the time. Dissolved oil at small concentrations could have been traveling by the Gulf Stream to the east coast of Florida and it is possible that this material was affecting microbial life in the Straits and producing slicks. There was also an upwelling event taking place on the Southeast Florida coast that was not present during the sampling event in 2013 (to view SST images indicating this event, please see http://marine.rutgers.edu/cool/sat_data/?bm=7&bd=10&by=2010&sort=date&em=7&ed=10&ey=2010&region=floridacoast&product=sst&nothumbs=0&okb.x=32&okb.y=21, for which Rutgers University allows public access). The sampling location in 2010 was also just north of a local sewage outfall, which could be affecting microbial populations as well.

5.3 Gulf of Mexico Comparisons

The addition of sample analysis from filters collected in the Gulf of Mexico was important to this study, as it allowed the expansion of regions sampled to see how this genus may differ in abundance in other parts of the ocean. In slick conditions on the 11th

of December, *Bacillus* abundance was not found to be statistically different in the subsurface samples compared to those collected from the microlayer (Fig. 22). There were no samples analyzed from non slick conditions to compare on this day. On the 16th, samples were collected in slick and non slick conditions, and there appeared to be a higher abundance in the slick microlayer samples. In fact, this area had the highest *Bacillus* abundance of any area compared in this Gulf of Mexico study. Conditions were calm at the sea surface, possibly leading to this distinct abundance in slick microlayer conditions. Samples collected the following day (17th) were collected much farther from shore than any other sampling area compared in this study. These samples clearly showed a very low abundance of *Bacillus*. Coastal areas are known to be rich in nutrients compared to areas farther out from land, and this finding seems to follow that assumption.

5.4 Variability between samples taken in the same location

An important finding from this study is that there is a very high variability in abundance levels between samples taken in the same location, making it difficult to determine any statistical differences between sampling areas. The level of variability can be due to both environmental conditions as well as sample processing and lab analysis. The ocean is a constantly changing environment, and microbial communities are at the mercy of these processes. When conditions change, all forms of marine life are affected. Events such as upwelling, algal blooms, and a change in weather can strongly affect community structure down to microbial life. Variability can also be from human error in the lab during extraction and PCR reaction setup. To combat this potential issue, extreme care was taken during extraction to minimize contamination from the researcher and lab

environment. Correct pipetting techniques were used during each PCR reaction setup and no expired materials were included in each master mix. Extreme care was also taken when handling temperature sensitive materials such as DNA Taq polymerase, which can lose its effectiveness if stored in certain temperature ranges.

6. Conclusions

It is important that microlayer sampling techniques become standardized across the scientific community to better compare results between studies (Cunliffe and Wurl 2014). There are currently a number of sampling methods used, all with their advantages and disadvantages. All vary significantly in sampling size, levels of potential contamination, and ease of use. The microlayer sampling method by Kurata (2012) and further advanced in this work should be considered as a universal microlayer sampling method. This technique minimizes contamination from the boat and boat wake as well as from the researchers. The lack of heavy equipment needed makes sampling easier and safer at the front of the boat and because each filter is only used once, there is no need to spend significant time cleaning a sampling device between collection events and after fieldwork is complete.

Two regions of the world's oceans were sampled and relative abundance levels of the surfactant associated genus *Bacillus* were determined using real time PCR techniques. This bacteria group was found in all locations and its abundance was highly variable in each area sampled. This variability is most likely due to the constantly changing marine environment as well as from slight differences in sample processing and analysis. In a number of cases, there were significant differences between the relative abundance of *Bacillus* between microlayer and subsurface samples collected. This may point to the

presence of distinct bacterial communities in these environments of close proximity. Differences in *Bacillus* abundance between the Straits of Florida and the Gulf of Mexico were difficult to determine, as there were a large range of abundance estimations in both locations. However, *Bacillus* appears to be more abundant closer to the coastline in the Gulf of Mexico, as the sampling location from 20 miles off shore in that region showed very little of these bacteria. An initial hypothesis was that surfactant associated bacteria in subsurface water may be playing a large role in the presence of sea slicks on the surface, however this study did not find any results to support this idea. No pattern was found regarding more abundance of *Bacillus* in the water column compared to the sea surface microlayer.

6.1 Limitations

A key limitation of this sampling method is the small amount of DNA collected from each filter. This amount makes it difficult to use DNA analysis techniques such as real time PCR and next generation sequencing technology. While other sampling methods such as the glass plate do produce a larger amount of sample to work with in the lab, there is also a high probability that subsurface water is also included.

Because of the small sample size of the filter sampling technique, a nested PCR stage had to be included in order to get any real time PCR results for the relative abundance of *Bacillus* in each sample. Nested PCR has been used in a number of studies, but doing so can create bias in the final results due to so many cycles of amplification. Any small errors will be further amplified using nested PCR.

The microbial communities found in the ocean are very complex and not fully understood. There are still many unknown species of marine bacteria and new types are constantly being discovered. For this study, genus identification was the lowest taxonomic group differentiated. The initial plan for this study was to determine the relative abundance of a number of potentially surfactant associated genera of bacteria. However, non specific amplification became an issue for a number of primer pairs and we were forced to focus on *Bacillus* for the remainder of the study.

6.2 Suggestions for future microbial microlayer studies

For future studies regarding microbial life found in the sea surface microlayer, it is suggested to increase the sample size to 10+ in order to address variability and obtain statistically representative results. It is also suggested that any samples to be compared should be extracted at the same time using a standard protocol and the same buffer solutions to ensure consistency. Taking the filters in and out of the freezer should be avoided if possible, as the refreezing process may lead to damage to the DNA samples. If nested PCR must be used during analysis, reducing the initial cycle number may help to avoid a leveling out effect, which would cause any differences in sequence abundance to disappear. To avoid the use of nested PCR, researchers may want to consider combining DNA extract from multiple filters taken in the same area to get more DNA per PCR sample. Future research should also focus on the chemical analysis of the materials found in slick microlayer conditions to determine their origin. Another option is to focus a new study on the presence of functional genes related to surfactant production. This study looked at gene sequences commonly used for taxonomy. While the certain genera of

bacteria listed earlier are known to be surfactant associated, certain species within those groups may not be producing surfactants.

The sea surface microlayer is an understudied environment that plays a big role in many processes at the air sea interface. The results of this study will aid in the improvement of this sampling technique, getting researchers closer to a method that can be used during all microlayer studies to compare their findings in the future.

7. References

Abraham, W.R., Meyer, H., Yakimov, M. (1998) Novel glycine containing glucolipids from the alkaneusing bacterium *Alcanivorax borkumensis*. *Biochem Biophys Acta* 1393:57-62.

Agogue, H., Casamayor, E.O., Bourrain, M., Obernosterer, I., Joux, F., Herndl, G.J., Lebaron, P. (2005a) A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FEMS *Microbiol Ecol* 54: 269–280.

Agogué H., Joux F., Obernosterer I., Lebaron P. (2005b) Resistance of marine bacterioneuston to solar radiation. *Appl. Environ. Microbiol.* 71, 5282–5289.

Banat I.M. (1995a) Biosurfactants characterization and use in pollution removal: state of the art. A Review. *Acta Biotechnol* 15:251–67.

Banat I.M. (1995b) Biosurfactants production and use in microbial enhanced oil recovery and pollution remediation: a review. *Bioresource Technology* 51:1–12.

Banat, I.M., Samarah, N., Murad, M., Horne, R., Banergee, S. (1991) Biosurfactant production and use in oil tank clean-up. *World Journal of Microbiology & Biotechnology* 7:80–8.

Baruzzi, F., Quintieri, L., Morea, M., Caputo, L., (2011) Antimicrobial Compounds Produced by *Bacillus spp*. and Applications in Food. In Science against Microbial Pathogens: Communicating Current Research and Technological Advances; Vilas, A.M., Ed.; Formatex pp. 1102–1111.

Cunliffe, M. and Wurl, O. (2014) Guide to Best Practices to Study The Ocean's Surface. http://www.scor-int.org/Publications/SCOR_GuideSeaSurface_2014.pdf

Cunliffe, M., Engel, A., Frka, S., Gašparović, B., Guitart, C., Murrell, J.C. (2013) Sea surface microlayers: A unified physicochemical and biological perspective of the air–ocean interface. *Prog Oceanogr* 109: 104–116.

Cunliffe, M., Upstill-Goddard, R. C. and Murrell, J. C. (2011) Microbiology of aquatic surface microlayers. *FEMS Microbiology Reviews* 35: 233–246.

Cunliffe, M. and Murrell, J.C. (2009) The sea surface microlayer is a gelatinous biofilm. ISME J 3: 1001–1003.

Cunliffe, M., Schafer, H., Harrison, E., Cleave, S., Upstill-Goddard, R.C., Murrell, J.C. (2008) Phylogenetic and functional gene analysis of the bacterial and archaeal communities associated with the surface microlayer of an estuary. ISME J 2: 776–789.

Gade, M., Alpers, W., Hühnerfuss, H., Wismann, V., and Lange, P.A. 1998. On the reduction of the radar backscatter by oceanic surface films: Scatterometer measurements and their theoretical interpretation. *Remote Sensing of Environment* 66:52–70

Galkiewicz, J.P. and Kellogg, C.A. (2008) Cross-Kingdom Amplification Using *Bacteria*-Specific Primers: Complications for Studies of Coral Microbial Ecology *Applied and Environmental Microbiology* 74: 7828-7831.

Garrett, W.D. (1965) Collection of slick-forming materials from the sea surface. *Limnol Oceanogr* 602-605.

Hamdache, A., Lamarti, A., Aleu, J., Collado, I.G., (2011) Non-peptide metabolites from the genus *Bacillus*. Journal of Natural Products 74:893–899.

Harayama, S., Kasai, Y., Hara, A. (2004) Microbial communities in oil-contaminated seawater. *Current Opinion in Biotechnology* 15:205–14.

Hardy, J.T. (1982) The sea-surface microlayer – biology, chemistry and anthropogenic enrichment. *Prog Oceanogr* 11: 307–328.

Higuchi, R., Dollinger, G., Walsh, P.S., Griffith, R. (1992) Simultaneous amplification and detection of specific DNA sequences. *Biotechnology* 10:413–417.

Liew, S.C. (2001) Principles of remote sensing. Centre for Remote Imaging, Sensing and Processing National University of Singapore.

Lindroos, A., Szabo, H.M., Nikinmaa, M., Leskinen, P. (2011) Comparison of sea surface microlayer and subsurface water bacterial communities in the Baltic Sea. *Aquat Microb Ecol* 65:29-42.

Liss, P.S. and Duce, R.A. (1997) The Sea Surface and Global Change. Cambridge Univ. Press, Cambridge.

Franklin, M. P., McDonald, I. R., Bourne, D. G., Owens, N. J. P., Upstill-Goddard, R. C. and Murrell, J. C. (2005) Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. *Environmental Microbiology*, 7: 723–736.

Kurata, N., Vella, K., Tartar, A., Matt, S., Shivji, M., Perrie, W., and Soloviev, A. 2015. Surfactant-associated bacteria in the near-surface layer of the ocean. *Nature Scientific Reports.* Manuscript in revision.

Kurata, N., Vella, K., Tartar, A., Matt, S., Shivji, M., Perrie, W., Soloviev, A. (2012) Surfactant-Associated Bacteria in the Sea Surface Microlayer and their Effect on Remote Sensing Technology. AGU Fall Meeting 2012. San Fransisco. Oral paper.

Kurata, N. (2012) Sea Surface Microlayer Microbial Observation System. Masters Thesis for Nova Southeastern University Oceanographic Center.

Maki, J.S. (2002) Neuston microbiology: life at the air-water interface. In: Bitton G (ed) The encyclopedia of environmental microbiology. Wiley, NewYork, NY, pp. 2133-2144.

Maneerat, S., Bamba, T., Harada, K., Kobayashi, A., Yamada, H., Kawai, F. (2006) A novel crude oil emulsifier secreted in the culture supernatant of a marine bacterium *Myroides sp*. Strain SM1. *Appl Microbiol Biotechnol* 70:254-9.

Mullis, K.B., and Faloona, F.A. (1987) Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. *Methods in Enzymology* 155: 335–350.

Mullis, K.B. (1990) The unusual origin of the polymerase chain reaction. *Scientific American* 262: 56–61.

Naumann, E. (1917) Beitrage zur kenntnis des teichnanoplantons. II. Uber das neuston des subwasser. *Biol Zentbl* 37:98-106.

Norkrans, B. (1980) Surface microlayers in aquatic environments. Advances in Microbial Ecology (Alexander M, ed), pp. 51–85. Plenum, New York.

Pace, N.R. (2009) Mapping the tree of life: progress and prospects. *Microbial Mol Biol Rev* 73:565-576.

Paul, V.J., Arthur, K.E., Williams, R.R., Ross, C., Sharp, K. (2007) Chemical defenses: From compounds to communities. *The Biological Bulletin* 213:226–251.

Perfumo, A., Banat, I.M., Canganella, F., Marchant, R. (2006) Rhamnolipid production by a novel thermotolerant hydrocarbon-degrading *Pseudomonas aeruginosa* AP02-1. J *Appl Microbiol Biotechnol* 72:132-8.

Powledge, T.M. (2004) The polymerase chain reaction. *Advances in Physiology Education* 28:44–50.

Rodrigues, L., Banat, I.M., Teixeira, G., Oliveira, O., (2006) Biosurfactants: potential applications in medicine. *Journal of Antimicrobial Chemotherapy* 57:609–18.

Satpute, S.K., Banat, I.M., Dhakephalkar, P.K., Banpurkar, A.G., Chopade, B.A. (2010) Biosurfactants, bioemulsifiers and exopolysaccharides from marine microorganisms. *Biotechnol. Adv*. 28:436-450.

Sayem, S.M.A., Manzo, E., Ciavatta, L., Tramice, A., Cordone, A., Zanfardino, A., de Felice, M., Varcamonti, M. (2011) Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of *Bacillus licheniformis*. *Microbial Cell Factories* 10, 74. 17.

Sieburth, J.M. (1983) Microbiological and organic-chemical processes in the surface and mixed layers. *Air-Sea Exchange of Gases and Particles* (Liss PS & Slinn WGN, eds), pp. 121-172. Hingham, MA.

Upstill-Goddard, R.C., Frost, T., Henry, G.R., Franklin, M., Murrell, J.C., Owens, N.J.P. (2003) Bacterioneuston control of air–watermethane exchange determined with a laboratory gas exchangetank.*Global Biogeochem Cy* 17: 19.11–19.15.

Valasek, M.A., and Repa, J.J. (2005) The power of real-time PCR. *Advances in Physiology Education* 29:151–159.

Vella, K. (2012) Microbial Effects on the Production and Transformation of Surfactants within the Microlayer and Subsurface Waters in Application to Remote Sensing Techniques. Masters Thesis for Nova Southeastern University Oceanographic Center.

Whitman, W.B.,Coleman D.C., Wiebe, W.J. (1998) Prokaryotes: The unseen majority. Proceedings of the National Academy of Sciences of the United States of America. 95:6578–6583.

Wurl, O., Wurl, E., Miller, L., Johnson, K., Vagle, S. (2011) Formation and global distribution of sea-surface microlayers. *Biogeosciences* 8: 121–135.

Wurl, O. and Holmes, M. (2008) The gelatinous nature of the sea-surface microlayer, *Mar. Chem.,* 110, 89–97.